HER2/Neu (ErbB2) Signaling to Rac1-Pak1 Is Temporally and Spatially Modulated by Transforming Growth Factor β

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Abstract
In HER2 (ErbB2)-overexpressing cells, transforming growth factor β (TGF-β), via activation of phosphoinositide-3 kinase (PI3K), recruits actin and actinin to HER2, which then colocalizes with Vav2, activated Rac1, and Pak1 at cell protrusions. This results in prolonged Rac1 activation, enhanced motility and invasiveness, Bad phosphorylation, uncoupling of Bad/Bcl-2, and enhanced cell survival. The recruitment of the HER2/Vav2/Rac1/Pak1/actin/actinin complex to lamellipodia was abrogated by actinin siRNAs, dominant-negative (dn) p85, gefitinib, and dn-Rac1 or dn-Pak1, suggesting that the reciprocal interplay of PI3K, HER2, and Rac GTPases with the actin cytoskeleton is necessary for TGF-β action in oncogene-overexpressing cells. Thus, by recruiting the actin skeleton, TGF-β "cross-links" this signaling complex at cell lamellipodia: this prolongs Rac1 activation and increases metastatic properties and survival of HER2-overexpressing cells. (Cancer Res 2006; 66(19): 9591-600)

Introduction
Transforming growth factor β (TGF-β) has been shown to synergize with transforming oncogenes in cancer progression (1). An oncogenic signaling network for which this has been shown is the ErbB family of receptor tyrosine kinases. Overexpression of active TGF-β1 or active mutants of the type I TGF-β receptor (Alk5) in the mammary gland of transgenic mice also expressing mouse mammary tumor virus-Neu (ErbB2) accelerates metastases from Neu-induced mammary cancers (2–4). In addition, a genetic modifier screen in nontumorigenic mammary epithelial cells identified TGF-β1 and TGF-β3 as molecules that cooperate with HER2 in inducing cell motility and invasion (5). Inhibition of HER2 with the HER2-neutralizing antibody trastuzumab blocked the promigratory effect of TGF-β1 on HER2-overexpressing mammary epithelial cells (6), suggesting that oncogene function is required for the transforming effect of TGF-β. Furthermore, TGF-β can activate signaling pathways downstream ErbB receptor tyrosine kinases such as Ras/mitogen-activated protein kinase (7) and phosphoinositide-3 kinase (PI3K)/Akt (8, 9). Inhibition of endogenous TGF-β function with dominant-negative (dn) type I TGF-β receptor has been shown to block growth of Ras-transformed tumors in vivo (10), suggesting that oncogenes can engage and, in turn, become dependent on TGF-β function for tumor progression. However, the molecular mechanisms of cross-talk between TGF-β and ErbB receptor signaling remain largely unknown.

The Rho family of small GTPases (RhoA, Rac1, and Cdc42) regulate the actin cytoskeleton and focal adhesion complexes, which are essential for morphogenesis, cell motility, and invasiveness (11). They also signal for cell proliferation and apoptosis through targeting of downstream effectors such as Pak, c-Jun NH2-terminal kinase (JNK), p38, Rock, signal transducers and activators of transcription 3, and nuclear factor-κB (NF-κB; ref. 12). Their activation is regulated by guanine nucleotide exchange factors, which catalyze the exchange of GDP for GTP and hence "switch on" the GTPase to its active GTP-bound form (13). GTPases like Rac1 have been shown to contribute to TGF-β-mediated cellular and transcriptional responses (14, 15). In addition, TGF-β can rapidly activate RhoA and Rac1, contributing to an epithelial to mesenchymal transition and enhanced cell motility (16, 17). Rac1 activity in situ is higher in mouse mammary cancers expressing Neu (ErbB2) and active TGF-β1 transgenes compared with transgenic tumors expressing the Neu oncogene alone (3). MCF10A human mammary epithelial cells that overexpress HER2/ErbB2 exhibit higher Rac1 activity than cells with low HER2 levels and this activity is blocked by treatment with a HER2-neutralizing antibody (6). Because HER2, as well as coreceptors in the ErbB family, can also activate Rac1 (18–20), these data suggest that, like PI3K/Akt, HER2/ErbB2 and TGF-β can converge at Rac1 and synergize for tumor progression.

We have examined mechanisms of TGF-β/Her2 interaction in MCF10A cells stably transduced with a HER2 vector and breast cancer lines that naturally overexpress the proto-oncogene. In these cells, treatment with TGF-β activated PI3K, leading to the recruitment of actin and actinin to HER2, which were colocaled with the Vav2 guanine nucleotide exchange factor, activated Rac1, and its effector Pak1 at cell protrusions. The stabilization of this complex at cell lamellipodia resulted in prolonged activation of Rac1, enhanced cell invasiveness, Bad phosphorylation at Ser136, uncoupling of Bad from Bcl-2, and enhanced cell survival.

Materials and Methods
Cells, plasmids, and viruses. Primary mammary tumor cells from Neu transgenic tumors were generated and maintained as described (2–4). MCF10A/VEC and MCF10A/HER2 cells were generated and maintained as previously described (6, 21). Phoenix-Ampho cells were grown in DMEM (Cambrex, Rockland, ME) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT) in a humidified 5% CO2 incubator at 37°C. Human breast tumor cell lines MCF7, MDA453, MDA361, and BT474 were purchased from the American Type Culture Collection (Manassas, VA) and maintained in IMEM/10% FBS. The following reagents were used: recombinant human TGF-β1 (R&D Systems, Minneapolis, MN), wortmannin (Sigma, St. Louis, MO, 0.1 μmol/L), and actinin siRNA (Dharmacon, Lafayette, CO). Cell proliferation was determined by the sulforhodamine-B method (CellTiter, Promega, Madison, WI). Cell motility was determined using a scratch assay (22). Cell adhesion was measured in boyden chambers with cell Migration Chambers (Neuro Probe, Bethesda, MD) using Matrigel (BD Biosciences, San Jose, CA) as described (23). Cell invasion was determined using a Matrigel invasion chamber (BD Biosciences) for 24 h, and the filter was stained with 0.1% crystal violet. The stained membrane was photographed and cellular invasion was determined by measuring the stained area in the filter using GeoMorph software (24). Cell apoptosis was determined with an annexin V–FITC apoptosis detection kit (BD Biosciences) and a FACSCalibur flow cytometer (BD Biosciences). Cell survival was determined with the sulforhodamine B dye (CellTiter) and a Perkin-Elmer Victor 1420 multilabel counter (Perkin-Elmer, Norwalk, CT). Cell cycle was determined with a BD LSR II flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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immunofluorescence staining of three-dimensional acini was done as previously described (24). Confocal analyses were done with Zeiss inverted LSM510 confocal microscopy system.

**Indirect immunofluorescence assay.** Immunofluorescence assay was done as previously described (21). For detection of GFP-bound Ras1, cells were fixed with methanol and incubated with 10 μg/mL GST-PBD protein (Cytoskeleton, Inc.) in 1% milk for 15 minutes and next with a GST antibody (Santa Cruz Biotechnology) for 1 hour at room temperature and with Oregon Green–a mouse secondary immunoglobulin G (IgG). Fluorescent images were captured using a Princeton Instruments (Trenton, NJ) cooled charge-coupled device digital camera from a Zeiss Axioshot upright microscope.

**Transfection of siRNA.** siRNA oligonucleotides designed to target the nucleotide sequence 5′-AAGTGCCACGTGAGGATCAC (for actinin) or 5′-AATCTGGAGCTGAGGATCAC (as a control sequence; mismatched nucleotides are underlined) were obtained from Qiagen (Valencia, CA). The siRNA oligonucleotides were transfected into MCF10A/HER2 cells grown on six-well plates or coverslips using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the procedure of the manufacturer. Expression of actinin was examined by immunoblot.

**Results.**

**TGF-β induces association of HER2 with the actin cytoskeleton.** To determine whether TGF-β–induced recruitment of proteins to the Neu transmembrane tyrosine kinase, we treated Neu-expressing primary mammary tumor cells (3) with TGF-β1 for 16 hours. Neu antibody or control IgG precipitations from these cells were separated by SDS-PAGE. Commissasie blue staining of the gel revealed 102-kDa and 47-kDa bands in the precipitates from treated but not from untreated cells (Fig. 1A). Mass spectrometry followed by database interrogation of the excised protein bands identified them as actinin-4, an actin-bundling protein that connects the actin cytoskeleton to the cell membrane (25), and actin, respectively. We next confirmed this association in MCF-10A human mammary epithelial cells stably expressing HER2 (ErbB2), the human homologue of Neu. By immunoprecipitation followed by immunoblot analysis, actin and actinin coprecipitated with HER2 in TGF-β–treated cells (Fig. 1B). Because TGF-β reorganizes the actin cytoskeleton and induces the formation of filamentous actin stress fibers, the association of HER2 with actinin suggested ligand-induced changes in the cellular distribution of HER2. Immunofluorescence staining of untreated cells revealed low levels of F-actin with HER2 distribution throughout the cell membrane. Treatment with TGF-β for 16 hours induced F-actin formation and colocalization of F-actin and HER2 predominantly at cell lamellipodia (Fig. 1C).

To determine if the association with actinin played a role in the recruitment of both HER2 and F-actin to cell protrusions, we knocked down the expression of actinin with siRNA oligonucleotides. In cells transfected with siRNA targeting actinin but not control siRNA, TGF-β–induced HER2 association with actinin and actin was abrogated (Fig. 1D, left). Consistent with this result, in cells transfected with actinin siRNA, TGF-β did not induce polymerization of F-actin or translocation of HER2 to lamellipodia (Fig. 1D, right).

**Rac1-Pak1 function is required for TGF-β–induced association of HER2 and the actin cytoskeleton.** We have previously reported that TGF-β rapidly increases the interaction of HER2 with Rac1 and Pak1 as well as Rac1 activity (6, 17). Pak1, a main effector of Rac1, interacts with cytoskeletal components and induces cytoskeletal reorganization (26). Therefore, we speculated that the association of HER2 and the actin cytoskeleton in TGF-β–treated...
cells also includes an interaction with Rac1 and Pak1. To test this, we examined if Pak1 associated with the HER2/actin/actinin complex in TGF-β-treated cells. TGF-β induced a time-dependent association of Pak1 with HER2, actin, and actinin, which was maximal at 8 hours (Fig. 2A). We next tested Rac1 and Cdc42 activities with GST-PBD fusion protein to capture the GTP-bound active form of Rac1 and Cdc42, followed by immunoblot with Rac1 and Cdc42 antibodies, respectively. Rac1 activity exhibited a biphasic response to TGF-β in MCF10A/HER2 cells but not in cells expressing vector alone with two peaks of activation at 4 and 24 hours (Fig. 2B). Cdc42 activity was not affected (data not shown). Increasing levels of HER2, actin, and actinin associated with GST-PBD as a function of time with treatment of TGF-β, suggesting an association of these with GTP-Rac1. In MCF10A/VEC cells, the kinetics of TGF-β–induced Rac1 activation were markedly slower with maximal stimulation at 8 hours and a more delayed association of actin and actinin with GST-PBD (Fig. 2B).

To determine if Rac1 and Pak1 are required for HER2 interactions with the actin cytoskeleton, we established MCF10A/HER2 cells stably expressing dn-Rac1 (Rac1-T17N; ref. 17), dn-Pak1 (Pak1-205; ref. 22), or pBabe vector. Precipitation from TGF-β–treated pBabe control cell lysates with Pak1, Rac1, or HER2 antibodies followed by immunoblot analyses indicated ligand-induced association of Pak1, Rac1, HER2, actin, and actinin. In cells expressing dn-Rac1 or dn-Pak1, this association was abrogated but ligand-induced phosphorylation of Smad2 was unaffected (Fig. 2C). Although at lower levels than in the presence of TGF-β, Rac1 and Pak1 were constitutively associated with HER2 (Fig. 2C, lane 1) and, interestingly, the basal phosphorylation of HER2 was markedly reduced in cells expressing dn-Rac1 and dn-Pak1 (Fig. 2C, lanes 7-12, first row). Finally, we examined if Rac1-Pak1 function is required for TGF-β–induced cell motility and adhesion. In cells transfected with vector alone, TGF-β accelerated wound closure and cell adhesion. These responses were impaired in MCF10A/HER2 cells expressing dn-Rac1 or dn-Pak1 (Fig. 2D and E), suggesting that Rac1-Pak1 function is required for TGF-β–induced cell motility and adhesion.

**HER2 phosphorylation is required for TGF-β–induced activation of Rac1 and HER2 translocation.** We then asked if the phosphorylation of HER2 is required to form complexes with Rac1, Pak1, and cytoskeletal components. We used the small-molecule ErbB tyrosine kinase inhibitor gefitinib, which in epidermal growth factor receptor (EGFR)–positive cells has been shown to inhibit HER2 phosphorylation (27). Treatment with gefitinib markedly reduced HER2 phosphorylation (Fig. 3A, third row). In addition, it reduced TGF-β–mediated activation of Rac1 and abolished ligand-induced association of HER2 with Pak1, Rac1, actin, and actinin (Fig. 3A), suggesting that activation of HER2 is necessary for its association with the actin cytoskeleton and the engagement of Rac1. Pretreatment with gefitinib did not prevent ligand-induced phosphorylation of Smad2, suggesting that the effect was ErbB specific and that TGF-β receptors were unaffected by the small-molecule inhibitor. Consistent with the coprecipitation experiments, preincubation with gefitinib prevented TGF-β–induced translocation of HER2 to lamellipodia as well as MCF10A/HER2 cell motility in a wound closure assay (Fig. 3B and C).
results imply that TGF-β requires a threshold of HER2 activation to engage Rac1-dependent cell motility.

**TGF-β and HER2 induce a PI3K-dependent interaction of Vav2 with Rac1.** Both activated TGF-β receptors and HER2 (28) have been shown to activate the PI3K/Akt pathway. Inhibition of PI3K has been shown to reverse a TGF-β-induced fibroblastoid phenotype and cell motility (29–31). In addition, PI3K has been shown to activate Rac1 and Pak1 (32–35) as well as the Rac guanine nucleotide exchange factor Vav2 (36). Because Vav2 has been shown to associate with ErbB receptors (19, 37, 38), we examined the role of PI3K and Vav2 in TGF-β–induced Rac1 activity in MCF10A/HER2 cells. Pretreatment with the PI3K inhibitor wortmannin prevented TGF-β–induced cell motility (not shown) and translocation of HER2 to lamellipodia (Fig. 4A). Subsequent immunoprecipitation experiments showed basal association of Vav2 and Rac1, which increased 1 hour after TGF-β treatment, corresponding temporally to an increase in Rac1 activity (Fig. 4B). These changes were still detectable in the presence of 100 μg/mL cycloheximide (data not shown). Like Rac1, HER2 associated constitutively with Vav2, but this association was not increased by treatment with TGF-β. Both basal and ligand-enhanced association of HER2 with Vav2 were prevented by gefitinib. On the other hand, neither gefitinib nor wortmannin reduced the constitutive basal Rac1/Vav2 but they both eliminated any enhanced association induced by TGF-β (Fig. 4B). This suggests that the HER2/Vav2 complex requires active HER2 but not PI3K. On the other hand, both HER2 and PI3K functions are permissive for Rac1/Vav2 complex formation.

The similar effects of gefitinib and wortmannin on the ligand-stimulated Rac1/Vav2 complex and on Rac1 activity suggested that the effects of the ErbB inhibitor were a result of PI3K inhibition. Thus, we examined the effect of gefitinib on PI3K catalytic activity as measured by the ability of immune complexes precipitated with p85 antibodies to stimulate formation of 3'-phosphorylated inositol lipids *in vitro*. In MCF10A/HER2 cells, TGF-β–activated PI3K within 1 hour after treatment. In the presence of gefitinib, both basal and ligand-induced PI3K activities were markedly reduced (Fig. 4C), implying that HER2 phosphorylation is permissive and/or required for TGF-β–stimulated PI3K activity. Consistent with this result, gefitinib and wortmannin each prevented ligand-mediated phosphorylation of Akt and Pak1 (Fig. 4B, bottom four rows). Finally, expression of adenoviruses encoding dn-p85 (AxΔp85; ref. 39) completely abolished the TGF-β–induced association of HER2 with actin and actinin, as well as Akt activation (Fig. 4D) and cell motility, in a wound closure assay (Fig. 4E), further suggesting a central role for PI3K in the crosstalk between TGF-β and HER2 signaling.

**TGF-β–induced HER2 translocation to lamellipodia recruits Vav2 and locally activates Rac1.** Inhibition of HER2 with gefitinib blocked TGF-β–induced recruitment of HER2 to cell lamellipodia, activation of Rac1 and PI3K, and the association of HER2 with Vav2. These data suggested that treatment with TGF-β results in HER2- and PI3K-dependent redistribution of HER2-associated Vav2.
Figure 3. Phosphorylation of HER2 is required for TGF-β–induced Rac1 activity and HER2 translocation. A, MCF10A/HER2 cells were serum starved overnight and pretreated with gefitinib (1 μmol/L) for 1 hour before adding TGF-β for 0 to 24 hours. Cell lysates were harvested for immunoprecipitation with Pak1 or HER2 antibodies or GST-PBD. The antibody or fusion protein pull-downs were subjected to immunoblot analysis with the antibodies shown on the right. Immunoblots of total cell lysates showed equal loading in each lane. Phosphorylation of HER2 and F-actin at cell protrusions only in BT474 cells (Fig. 5C). Finally, TGF-β–induced invasion into Matrigel 1.6-fold. Ligand-induced invasion was completely blocked in cells expressing dn-Rac1 or dn-Pak1 (Fig. 5C).

We next studied if the effects of TGF-β on cell invasiveness correlated with the levels of HER2 in a panel of human breast cancer cell lines. MCF7 cells underexpress HER2. MDA453 and MDA361 overexpress HER2 protein but do not exhibit gene amplification, whereas in BT474, the HER2 gene is amplified (42). In all lines, except MCF7, treatment with TGF-β enhanced cancer cell invasion through Matrigel; this increase was 40% to 60% for MDA453 and MDA361 and 180% for BT474 cells (Fig. 5D). A similar trend was observed in terms of the ability of TGF-β to induce association of HER2 with cytoskeletal elements. Both basal and ligand-stimulated association of HER2 with actin and actinin were absent in MCF7, detectable in MDA453 and MDA361, and highest in BT474 cells (Fig. 5E). Finally, TGF-β–induced colocalization of HER2 and F-actin at cell protrusions only in BT474 cells (Fig. 5F), implying that a threshold of HER2 is required for TGF-β–stimulated cell invasiveness.

Rac1-Pak1 function is required for TGF-β–induced cell survival in HER2-overexpressing cells. The Rac1-Pak1 pathway has been shown to regulate cell survival by down-regulating proapoptotic pathways (43, 44). TGF-β protects tumor cells from serum starvation– or detachment-induced apoptosis (anoikis; refs. 23, 29, 45, 46). Thus, we next examined if Rac1-Pak1 are involved in TGF-β–mediated protection from anoikis using an Apo-BrdUrd assay in suspended cells expressing dn-Rac1, dn-Pak1, or vector alone. Compared with adherent cells, suspended MCF10A/HER2 control cells showed 38% apoptosis, which could be partially protected by adding TGF-β. In cells stably expressing dn-Rac1 or dn-Pak1, TGF-β failed to protect cells from anoikis (Fig. 6A). Consistent with this result, cells suspended in medium containing TGF-β exhibited lower levels of cleaved caspase-3 and DNA to lamellipodia, which in turn regulates the local activation of Rac1. To test this possibility, we double-stained HER2 and Vav2 in TGF-β–treated MCF10A/HER2 cells. To localize Rac1 activation, we incubated fixed cells with GST-PBD and then with a GST antibody to detect GTP-bound Rac1. In ligand-stimulated cells, HER2 colocalized with Vav2 and with activated Rac1 at cell lamellipodia (Supplementary Fig. S1A and B). Because GST-PBD also recognizes Cdc42, we cannot completely rule out the involvement of this GTPase although it is known to trigger filopodial extensions. The colocalization of HER2 and activated Rac1 was abolished in cells treated with gefitinib orwortmannin as well as in cells expressing dn-Rac1, dn-Pak1, or dn-p85 (Supplementary Fig. S1C), implying that both HER2 phosphorylation and PI3K are required for Vav2 recruitment and local Rac1 activation.

TGF-β and HER2 cooperate in tumor cell invasiveness. TGF-β and HER2 have been shown to synergize in tumor metastasis. Thus, we examined whether they also cooperated in tumor cell invasiveness via Rac1-Pak1 ex vivo. MCF10A cells form polarized, quiescent acini-like spheroids in three-dimensional basement membrane gels. Activation of HER2 in these cells has been shown to reinitiate proliferation, disrupt tight junctions and apical polarity, and induce acinar expansion without invasion of the surrounding matrix (40). TGF-β treatment of MCF10A/HER2 cells induced three-dimensional acinar expansion and invasive protrusions into the surrounding Matrigel (Fig. 5A). To investigate branching morphogenesis, we used a mixture of Matrigel/collagen I, which has intermediate stiffness that is optimal for cell invasion compared with a softer matrix such as Matrigel (41). In a matrix containing 2 mg/mL collagen I (a 1:1 mixture of Matrigel/collagen I), the MCF10A/HER2 cells exhibited an elongated shape not observed in the vector controls. Addition of TGF-β to the HER2-overexpressing cells enhanced their elongated fibroblastoid shape and arranged them as invasive cords into the surrounding matrix; these effects were abrogated in cells expressing dn-Rac1 or dn-Pak1 (Fig. 5A). In MCF10A/HER2 cells within the TGF-β–stimulated invasive cords, F-actin was induced and it colocalized with HER2 as measured by immunofluorescence (Fig. 5B). Finally, we tested the ability of the cells to invade through Matrigel-coated chambers. In MCF10A/HER2 cells, but not in control cells, TGF-β increased invasion into Matrigel 1.6-fold. Ligand-induced invasion was completely blocked in cells expressing dn-Rac1 or dn-Pak1 (Fig. 5C).
Pak1 have been reported to induce Bad phosphorylation at Ser112. Induced Rac1 activation does not require cell adhesion. Rac1 and Pak1 have been reported to induce Bad phosphorylation at Ser112 and Ser136, leading to reduced interaction with Bcl-2 and thus fragmentations in agarose gels, but this was not observed when dn-Rac1 or dn-Pak1 was expressed (Fig. 6B).

We next examined GTP-bound Rac1 levels over time in cells suspended in medium with or without TGF-β. Cells in medium containing TGF-β exhibited an increase in GTP-bound Rac1 levels as early as 1 hour on suspension (Fig. 6C), suggesting that ligand-induced Rac1 activation does not require cell adhesion. Rac1 and Pak1 have been reported to induce Bad phosphorylation at Ser112 and Ser136, leading to reduced interaction with Bcl-2 and thus enhanced survival and Ser136. Phosphorylation of Bad was induced by TGF-β in suspended MCF10A/HER2 cells expressing pBabe vector alone but not in cells expressing dn-Rac1 or dn-Pak1. This increase of Bad phosphorylation in TGF-β–treated cells correlated temporally with a decrease of Bad-associated Bcl-2 (Fig. 6D). As expected, both dn-Rac1 and dn-Pak1 inhibited ligand-stimulated Pak1 phosphorylation in Thr423, which corresponds to an autophosphorylation site in the activation loop of its kinase domain (26). However, they did not affect basal or TGF-β–stimulated Ser112 Akt phosphorylation (Fig. 6D, seventh row) or PI3K activity (data not shown), suggesting that Rac1-Pak1 are downstream of activated PI3K.

Discussion

There is increasing evidence that in advanced cancers, TGF-β predominantly contributes to tumor progression. Although this outcome may involve TGF-β–mediated paracrine effects that modulate the tumor microenvironment and the host immune system, other data indicate that the effects of TGF-β on the tumor cells themselves have an essential role in cancer cell viability and progression (47). In addition to activating the Smad tumor suppressor pathway, TGF-β can engage signaling programs associated with transformation such as Ras/MAPK, PI3K/Akt, Rho GTPases, NF-κB, and JNK (8, 48, 49). In some cases, blockade of these pathways abrogates the transforming effects of TGF-β.

For example, MAPK and PI3K inhibitors block TGF-β–induced motility in oncogene-overexpressing mammary epithelial cells (5, 6). TGF-β–induced epithelial to mesenchymal transition, protection from apoptosis, and/or tumor cell migration is blocked by the PI3K inhibitor LY294002 or with expression of dn-Akt (23, 29–31, 46). The ErbB receptor network, which includes the HER2/Neu (ErbB2) tyrosine kinase, has been shown to synergize with TGF-β in transformation (see Introduction). Molecular mechanisms of ErbB/TGF-β cooperation that have been proposed include (i) differential transcriptional modulation in cooperation with Smads; (ii) activation of Smad-independent signal transducers, such as those listed above; (iii) attenuation of TGF-β–mediated, Smad-dependent antiapoptotic effects via up-regulation of Smad7; and (iv) autocrine induction of ligands that activate ErbB tyrosine kinases (50).
We have shown that in HER2-overexpressing mammary epithelial cells, TGF-β, via activation of PI3K, recruits the actin cytoskeleton to HER2, which then colocalizes with Vav2, activated Rac1, and its effector Pak1 at cell lamellipodia, leading to prolonged Rac1 activation and enhanced cell motility and survival. These effects occurred in the presence of cycloheximide as well as in suspended cells, suggesting that they did not require gene expression and/or new protein synthesis or cell adhesion. As such, they represent a novel mechanism of ErbB/TGF-β/cytoskeleton signaling cross-talk that may not invoke transcriptional effects.

It is generally accepted that in cells with low HER2 levels, the receptor is unable to generate a transforming signal. Studies with natural and transfected cell lines and primary human tumors that overexpress HER2 have shown ligand-independent phosphorylation of HER2, as shown in this study in MCF10A/HER2 cells. The biochemical basis of this constitutive phosphorylation is not clear but concurs with the reported ability of wild-type Neu to spontaneously multimerize and become activated when present in cells at high density (51). Another possible mechanism of HER2 phosphorylation is via ligand-induced activation of EGFR (ErbB1), ErbB3, or ErbB4 (28). Indeed, in MCF10A/HER2 cells, HER2 was constitutively phosphorylated and associated with Rac1, Pak1, Vav2, actin, and actinin. This resulted in activation of PI3K/Akt in the absence of added ligands. Interestingly, inhibition of Rac1 and...
Pak1 with dominant-negative constructs markedly reduced basal HER2 phosphorylation without affecting HER2 protein levels (Fig. 2C), suggesting that Rac1-Pak1 are permissive for ligand-independent HER2 activity. This is consistent with the role of Rac1-Pak1 in transformation induced by other oncogenes (33, 52). However, the magnitude and stability of these protein-protein associations and signaling output in the absence of TGF-β were not above a threshold necessary to recruit the HER2/Vav2/Rac1/Pak1/actin/actinin complex to the leading edge of the cell to initiate and maintain cell motility. By recruiting the actin skeleton, TGF-β stabilizes and/or "cross-links" this complex at cell lamellipodia, thus prolonging Rac1 activation (Fig. 7). In turn, this localization of HER2 at membrane protrusions may further enhance its resistance for internalization and prolong signaling (53).

The recruitment of the HER2/Vav2/Rac1/Pak1/actin/actinin complex to cell protrusions and cell motility were abrogated by the inhibition of PI3K with either dn-p85 or wortmannin, by inhibition of HER2 phosphorylation with gefitinib, by stable expression of either dn-Rac1 or dn-Pak1, and by RNAi against actinin, suggesting that the reciprocal interplay of PI3K, HER2 kinase, and Rac GTPase activities with the actin cytoskeleton is necessary for TGF-β action in oncogene-overexpressing cells. Treatment with wortmannin blocked TGF-β-induced activation of Rac1 and its association with Vav2, but expression of dn-Rac1 and dn-Pak1 did not affect ligand-induced p-Akt or PI3K catalytic activity, suggesting that Rac1-Pak1 are downstream of PI3K. Similar data have been reported by Wilkes et al. (9, 54) in fibroblasts where TGF-β can induce PI3K-dependent and Akt-independent Pak2 activity. We speculate, however, that PI3K-dependent, locally activated Rac1 may mediate the accumulation of phosphatidylinositol-3,4,5 trisphosphate and actin polymers at membrane protrusions as it has been shown in nontransformed cell systems (55, 56). This speculation requires further investigation.

Figure 6. Rac1-Pak1 pathway is required for TGF-β-induced cell survival. A, MCF10A/HER2 cells expressing dn-Rac1, dn-Pak1, or pBabe vector were suspended and incubated in serum-free medium with or without TGF-β for 16 hours, followed by Apo-BrdUrd assay. FL3-H images show propidium iodide staining for DNA content. The mean percentage ± SD of apoptotic (gated) cells from three experiments is indicated. Top row, similar number of cells that were allowed to attach on a 100-mm culture dish for 16 hours in serum-free medium. B, the same cell lines were trypsinized and resuspended in serum-free medium with or without TGF-β. Cells were collected at 0 to 16 hours after resuspension and prepared for caspase-3 immunoblot analysis (top) or DNA laddering in agarose gels (bottom). C, MCF10A/HER2 cells were trypsinized and resuspended in serum-free medium with or without TGF-β. Cells were harvested at 0 to 8 hours after resuspension and lyses prepared for GST-PBD pull-down and immunoblot with a Rac1 antibody. D, MCF10A/HER2 cells expressing dn-Rac1, dn-Pak1, or pBabe vector were trypsinized and resuspended in serum-free medium with or without TGF-β. Cells were harvested 8 hours after resuspension and cell lysates prepared for immunoblot analyses with the indicated antibodies. The level of Bad-associated Bcl-2 was determined by immunoprecipitation with a Bad antibody followed by Bcl-2 immunoblot.

Figure 7. Temporal and spatial modulation of HER2 signaling to Rac1-Pak1 by TGF-β. In cells with low HER2 levels, the receptor is not activated or associated with signal transducers or the actin cytoskeleton (A). In cells with HER2 gene amplification, HER2 is constitutively phosphorylated and associated with PI3K, Rac1, Pak1, Vav2, actin, and actinin. Although this complex may be enough to generate survival or proliferative signals, because it is not enriched at cell protrusions, it cannot induce cell motility (B). In cells with overexpressed and activated HER2, TGF-β induces PI3K above a necessary threshold required for the recruitment and stabilization of F-actin and the HER2 complex at cell protrusions, thus leading to prolonged Rac1 activation, cell movement, and increased survival on cell detachment (C).
Both Rac1 and Pak1 have been shown to suppress apoptosis (57–59). Rac1 stimulates Bad phosphorylation on Ser15, thereby suppressing drug-induced caspase apoptosis and apoptosis in human lymphoma cells (44). Pak1 can also phosphorylate Bad in vitro and in vivo on Ser112 and Ser136 (45). Pak1 also contributes to cell survival via phosphorylation of the forkhead transcription factor FKHR, leading to the exclusion of FKHR from the nucleus (60). Akt can stimulate Pak1 through a GTPase-independent mechanism and, in turn, induce Bad phosphorylation and enhance survival, which can be abolished by dn-Pak1 (33). Levels of p-Akt were similar in MCF10A/HER2 cells expressing dn-Rac1, dn-Pak1, and vector alone either in the presence or absence of TGF-β1. TGF-β1 prevented anoikis, induced phosphorylation of Bad at Ser136, and uncoupled Bcl-2 from Bad in control cells but not in those expressing dn-Rac1 or dn-Pak1 (Fig. 6D). These data suggest that TGF-β1 and HER2 synergize for enhanced tumour cell survival via PI3K and Rac1-Pak1 and that Akt is not sufficient to protect these cells from anoikis.

In summary, TGF-β1 modulates HER2 signaling to Rac1-Pak1 by compartmentalizing a HER2/Vav2/Rac1/Pak1/aktin/actin complex to the cell lamellipodia. This results in enhanced cell motility and survival. If operative in cancer cells, this mechanism would enhance tumor cell dissemination from primary tumor sites and their subsequent metastatic progression. In addition, this cross-talk suggests a rationale for the combined use of HER2 and TGF-β1 inhibitors in cancer patients with HER2 overexpression and evidence of active TGF-β1 signaling.

Acknowledgments

Received 6/6/2006; revised 7/21/2006; accepted 8/16/2006.

Grant support: NIH R01 grants CA52212 and CA19815 (C.L. Arteaga); Breast Cancer Specialized Program of Research Excellence grant P50 CA98131, and Vanderbilt-Ingram Comprehensive Cancer Center Support grant P30 CA68485.

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We thank Drs. Shimian Qu, Jae Youn Yi, and Yasuhiro Koh for helpful comments and Teresa Daggar for administrative and technical support.

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