Tiam1 Overexpression Potentiates Heregulin-induced Lymphoid Enhancer Factor-1/β-Catenin Nuclear Signaling in Breast Cancer Cells by Modulating the Intercellular Stability*

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Heregulin-β1 (HRG) promotes motility, scattering, and invasiveness of breast cancer cells. Tiam1, a newly identified guanine nucleotide exchange factor, has been shown to inhibit or promote cell migration in a cell type-dependent manner. In this study, we identified Tiam1 as a target of HRG signaling. HRG stimulation of breast cancer epithelial cells induced the phosphorylation and redistribution of Tiam1 to the membrane ruffles and the loosening of intercellular junctions. In addition, HRG-mediated scattering of breast epithelial cells was accompanied by stimulation of tyrosine phosphorylation and redistribution of β-catenin from the cell junctions to the cytosol and, finally, entry into the nucleus. Decompaction of breast cancer epithelial cells by HRG was accompanied by a transient physical association of the tyrosine-phosphorylated β-catenin with the activated human epidermal growth factor receptor 2 and subsequent nuclear translocation of β-catenin, as well as β-catenin-dependent transactivation of T-cell factor/lymphoid enhancer factor-1. All of these HRG-induced phenotypic changes were regulated in a phosphatidylinositol-3 kinase-sensitive manner. HRG-induced cellular ruffles, loss of intercellular adhesiveness, and increased cell migration could be mimicked by overexpression of a fully functional Tiam1 construct. Furthermore, ectopic expression of Tiam1 or of an active β-catenin mutant led to potentiation of the β-catenin-dependent T-cell factor/lymphoid enhancer factor-1 transactivation and invasiveness of HRG-treated cells. We also found preliminary evidence suggesting a close correlation between the status of Tiam1 expression and invasiveness of human breast tumor cells with the degree of progression of breast tumors. Together, these findings suggest that HRG regulate Tiam1 activation and lymphoid enhancer factor/β-catenin nuclear signaling via phosphatidylinositol-3 kinase in breast cancer cells.

The exposure of cells to growth factors has been shown to cause cytoskeletal reorganization, formation of lamellipodia, membrane ruffling, altered cell morphology, and increased cell migration and invasion (1). Most eukaryotic cells can migrate over or through a substrate, and cell motility plays a key role in both normal and pathological cellular physiology, the latter exemplified by invasion and metastasis (2). In many tissues, cells are stationary, but appropriate stimuli or oncogenic transformation or both can activate motility. One of the earliest responses of cells to many extracellular growth factors is the rapid reorganization of their cytoskeleton and cell shape.

Growth factors and their receptor interactions play an essential role in the regulation of epithelial cell proliferation, and abnormalities in growth factor expression and action may contribute to the progression and maintenance of the malignant phenotype. For example, HER2 overexpression is frequently associated with an aggressive clinical course, shorter disease-free survival time, poor prognosis, and increased metastasis in human breast cancer (3, 4). In addition, accumulating evidence suggests that the progression of human breast cancer cells may be influenced by HRG, a combinatorial ligand for HER3 and HER4 receptors (4). Recently, we and others have demonstrated that HRG activation of breast cancer cells promotes the development of more aggressive phenotypes in breast cancer cells (5, 6). The activation of HRG-signaling pathways (7, 8) has also been linked to the progression of breast cancer cells to a more invasive phenotype. These observations suggest that ligand-driven activation of HER receptors may play an important biological role in the progression of breast cancer cells to a malignant phenotype. HRG stimulation of breast cancer cells enhances activation of PI 3-kinase, MAP kinases, and p38 MAP kinases (5, 7–10), whereas the nature of the pathways by which HRG signals reorganize cell to cell junctions and its molecular implication remains poorly understood.

Cell-cell adhesive functions are dynamic in various contexts, including the establishment of epithelial cell polarity, compaction of the early embryo, scattering, and tumorigenesis (11, 12). Cadherins are single transmembrane-spanning proteins, containing an extracellular domain (N terminus) composed of five similar repeats (13). The homotypic binding between such domains on adjacent cells promotes the formation of higher order cadherin complexes effecting cell-cell adhesion. Several cytosolic proteins, including β-catenin, have been shown to bind to highly conserved amino acid sequences within the intracellular domain of E-cadherin (14, 15). Another cytosolic protein, α-catenin, connects the cadherin-catenin complex via β-catenin to actin filaments either directly or through α-actinin (16). According to a three-stage model of cell-cell adhesion formation
in polarized epithelial cells, stabilization of actin via the clustered cadherin-catenin complex engages the myosin II clutch, thereby inducing translocation of circumferential actin cables from the rest of the cell body to the cell-cell contact interface with final establishment of strong compacted cell-cell contacts (17, 18). However, there is no accepted model to define the reversed series of events leading to cell scattering or cell mass decompaction.

Cell-cell adhesion is a dynamic process in which the interactions between cell adhesion molecules and the cytoskeleton are likely to be modified. It is generally believed that phosphorylation and dephosphorylation of cadherins and catenins by receptor tyrosine kinases play an important role in this process. Hazan and Norton (19) showed that epidermal growth factor induces a dissociation of E-cadherin and β-catenin from α-catenin and hence from actin. Muller et al. (20) have shown that increasing β-catenin tyrosine phosphorylation correlates with the dissociation of β-catenin from the cadherin complex and an increase in the free cytoplasmic pool of β-catenin, suggesting that phosphorylation of β-catenin at the adherens junctions could be a mechanism for regulating the migratory and invasive behavior of the cell (20). Furthermore, data from Drosophila and Xenopus models (21–25) suggested an additional function for β-catenin independent of cadherin-mediated cell adhesion. This involves translocation of β-catenin to the nucleus that is preceded by its accumulation in the cytoplasm. The signals resulting in a free stabilized pool of β-catenin have further been correlated with tumor formation (17, 26, 27).

The small GTPases of the Rho family have been recognized as key regulators of signal transduction pathways that mediate the distinct actin cytoskeleton changes required for cell spreading, chemotaxis, or invasion (1). Rho-like GTPases, in conjunction with guanine nucleotide exchange factors (GEFs), function as molecular switches by cycling between an active GTP-bound state and an inactive GDP-bound state. Several Cdc42 and Rac1 effectors have been shown to regulate the E-cadherin-mediated adhesive activity mainly by acting on the actin cytoskeleton or the cadherin-catenin complexes (28, 29). GEFs such as Tiam1, which activates GTPase, have been shown to produce cytoskeletal changes similar to those induced by Rac1 (30–32). The biological effects of Tiam1 appear to be cell type-specific. For example, Tiam1 promotes the formation of E-cadherin-mediated cell-cell adhesion and thus inhibition of epithelial cell migration (31). In addition, Tiam1 has also been shown to promote migration of epithelial cells cultured on collagen (30, 31). However, very little is known about the mechanism by which Tiam1 induces de-adhesion of cells, particularly the Tiam1 function in breast cancer cells. In this study, we examined the influence of HRG signaling on the modifications of the components of the adherens junctions and investigated the potential role of Tiam1 in the activation of β-catenin nuclear signaling.

MATERIALS AND METHODS

Cell Cultures and Reagents—MCF-7 human breast cancer cells (5) were maintained in Dulbecco’s modified Eagle’s medium/F12 (1:1) supplemented with 10% fetal calf serum. Kinase inhibitors PD98059, SB203580, and LY294002 were from Calbiochem. Tiam1 and LEF1 antibodies were from Santa Cruz Biotechnology. Antibodies against E-cadherin and β-catenin were obtained from Zymed Laboratories Inc. [γ-32P]ATP was from PerkinElmer Life Sciences. C580-Tiam1 and CT199-Tiam1 were generously provided by Dr. J. Collard and have been described previously (31, 33), mutant β-catenin, or LEF1 mutants, as well as the dominant-negative p85 subunit of PI 3-kinase complex, AKT, MAP kinase, and p38 MAP kinase constructs, have been described in earlier publications (10, 34, 35).

Reporter Gene Assays—MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium/F12 supplemented with 10% fetal calf serum. Transient transfections were performed using Fugene-6 from Roche Molecular Biochemicals. In brief, exponentially growing cells were cultured in six-well plates and transfected with 0.5 μg of reporter, 0.02 μg of pCMVβGal control, and 0.5 μg of different effector constructs. 48 h after transfection, the cells were shifted to medium containing 1% fetal calf serum and treated with HRG (30 ng/ml) with or without inhibitors as needed. Luciferase assays were performed 48 h after transfection and normalized through β-galactosidase activity. Each assay was performed in triplicate sets at least three times.

In Vivo Phosphorylation Assays and Immunoprecipitation—MCF-7 cells were equilibrium labeled with [32P]orthophosphoric acid for 10 h and treated with HRG and/or pretreated with different chemical inhibitors as indicated. Labeled Tiam1 and β-catenin were immunoprecipitated with the desired antibodies, and the material was loaded on a 7% SDS-polyacrylamide gel electrophoresis gel, dried, and exposed, and the bands were quantified using a PhosphorImager.

Indirect Immunofluorescence—MCF-7 cells grown on coverslips were treated for 30 min with chemical inhibitors, followed by HRG (30 ng/ml) for another 15 or 30 min as desired. Cells were fixed with 3.8% paraformaldehyde for 10 min, followed by chilled acetone for 2 min. After being washed with phosphate-buffered saline, the cells were incubated with HECID-1 Ab, anti-β-catenin Ab, anti-HA Ab, anti-LEF1 Ab, or anti-Tiam1 diluted in phosphate-buffered saline for 60 min at room temperature. After three washings with phosphate-buffered saline, the cells were incubated with fluorescein isothiocyanate- or Alexa 546-labeled secondary antibodies or TOPRO-3 for DNA staining (Molecular Probes) for 45 min at room temperature. Where cells were co-stained for F-actin and Tiam1, Alexa 546-phalloidin was added, together with the fluorescein isothiocyanate-labeled secondary antibody.

Invasion Assays and Migration Assays—To measure the migration potential of cells overexpressing Tiam1 constructs, cells were co-transfected with each of the effector constructs (pcDNA3, C1199-Tiam1, C580-Tiam1, or β-catenin mutant) along with a GFP-containing construct, using the electroporation method and plated on the upper side of the trans-well filter device. Cells were immediately treated with HRG and allowed to migrate across the filter and invade the thick layer of Matrigel covering its lower side. After 12 h, the filters were removed, fixed, and stained using propidium iodide DNA-specific dye (Sigma). Stained filters were mounted on microscope slides and analyzed by confocal microscopy for GFP-positive cells on both sides of the filter. The results show the percentage of GFP-positive cells that invaded the filter from total GFP-positive cells counted on both sides of the filter. Because all the cells (including the GFP-negative cells) were counted, the overall transfection efficiency was also estimated to be between 20 to 30%.

RESULTS AND DISCUSSION

HRG Induces Tiam1 and β-Catenin Phosphorylation, along with Their Subcellular Relocalization—To investigate the possible participation of Tiam1 in HRG-mediated cytoskeleton modifications, we first examined the effect of HRG on the distribution of Tiam1. MCF-7 cells were treated with or without HRG, co-stained for E-cadherin/Tiam1 (Fig. 1A) and F-actin/Tiam1 (Fig. 1B), and analyzed using confocal microscopy. Control polygonal cells, growing as compact clusters, displayed an intercellular membranous co-localization of Tiam1 with E-cadherin (Fig. 1A, upper panels) or F-actin (Fig. 1B, left panel). After 15 min of HRG treatment, a significant amount of Tiam1 became polarized, showing a general tendency to migrate from the cluster in HRG-treated cells, known as the scattering effect induced by HRG in MCF-7 cells and analyzed in detail as described previously (5, 10). The observed ruffles induction (Fig. 1, A and B) preceding the decompaction effect in HRG-treated MCF-7 cells was also accompanied by a significant increase of Tiam1 phosphorylation (Fig. 1C, upper panel). To perform this experiment, MCF-7 cells were metabolically labeled with [32P]orthophosphoric acid, and cell lysates were

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immunoprecipitated with anti-Tiam1 antibody. Another major component of cadherin-rich intercellular junctions is \( \beta \)-catenin, which may be a target of HRG signaling. Accordingly, we examined the HRG regulation of \( \beta \)-catenin phosphorylation. HRG treatment significantly stimulated \( \beta \)-catenin phosphorylation (Fig. 1C).

Data from the literature suggest that the cytoplasmic pool of \( \beta \)-catenin might increase the level of the nuclear pool of \( \beta \)-catenin in some systems (15, 17, 20). To examine whether HRG signaling results in increased nuclear translocation of \( \beta \)-catenin, we co-stained control and HRG-activated (30 min) cells with E-cadherin- and \( \beta \)-catenin-specific antibodies and analyzed by confocal microscopy (Fig. 1D). We found that in addition to an increase within the cytoplasm, there was a significant enhancement of \( \beta \)-catenin immunostaining inside the nucleus of HRG-treated cells (Fig. 1, lower panel). This was accompanied by cell-shape changes with appearance of some \( \beta \)-catenin and E-cadherin immunoreactivity at the cellular free edges (Fig. 1D, arrows). In contrast, in control untreated cells, \( \beta \)-catenin was almost exclusively restricted at the level of intercellular junctions (Fig. 1D, upper panel).

**HRG Stimulates Tyrosine Phosphorylation of \( \beta \)-Catenin through the PI 3-Kinase Pathway and Its Association with HER2**—Because HRG has been shown to activate several signaling pathways (4, 5, 7, 10, 36), we next examined the nature of the signaling pathway responsible for HRG-induced \( \beta \)-catenin phosphorylation. Our results showed that the HRG-increased phosphorylation status of the immunoprecipitated \( \beta \)-catenin was completely reduced by blocking the PI 3-kinase pathway by pharmacological inhibitor LY294002 and only modestly reduced by inhibition of the MAP kinase pathway with the chemical inhibitor PD98059 (Fig. 2A).

Previously, epidermal growth factor receptor was shown to be physically associated with \( \beta \)-catenin (37). To investigate the potential interaction of Tiam1, HER2, and \( \beta \)-catenin, cell lysates were immunoprecipitated with an anti-HER2 antibody,
and the precipitated material was immunoblotted with different antibodies. We found no evidence of an association of Tiam1 with HER2 receptors (Fig. 2B). In contrast, there was a significant increase in the content of β-catenin associated with activated HER2 receptor (Fig. 2B). Thus, reprobing the above blot with a tyrosine-specific antibody revealed that besides HER2-increased tyrosine phosphorylation, HRG treatment also promoted the level of tyrosine-phosphorylated β-catenin associated with HER2, with a maximum effect detected at 15 min (Fig. 2B, lowest panel).

To further investigate the signaling pathway responsible for the association between the HER2 and β-catenin, MCF-7 cells were treated for 15 min, with or without HRG in the presence of specific chemical inhibitors of PI 3-kinase (LY294002), p38 MAP kinase (SB203580), or MAP kinase (PD98059). Cell lysates were immunoprecipitated with an anti-HER2 antibody and immunoblotted with β-catenin. The association between the two proteins was completely blocked by the PI 3-kinase pathway chemical inhibitor or partially reduced by suppressing the MAP kinase pathway. No blocking effect could be seen after blocking the p38 MAP kinase pathway. This analysis indicated that a functional PI 3-kinase pathway may be preferentially required for both phosphorylation of β-catenin and its association with HER2 upon HRG signaling.

HRG-induced LEF/β-Catenin Transactivation and Ruffles Formation Are PI 3-Kinase Pathway-dependent Events—To understand the significance of β-catenin nuclear translocation we further investigated the influence of HRG-inducible signaling pathways to support the transactivation from the LEF/TCF promoter. LEF1 is a transcription factor that cannot stimulate transcription of its target genes by itself but can augment promoter activity in association with β-catenin/TCF signaling (18, 26). To examine whether HRG-induced membrane dynamics and β-catenin nuclear translocation lead to increased β-catenin/TCF-LEF signaling, we examined the effect of HRG on the transcriptional activation from a synthetic LEF/β-catenin responsive promoter construct containing three LEF/TCF binding sites. As shown in Fig. 3A, suppression of PI 3-kinase by LY294002 or a dominant-negative mutant of the p85 subunit of PI 3-kinase (5), as well as its downstream effector AKT, was accompanied by blockade of β-catenin nuclear signaling-

Fig. 2. Analysis of signaling pathways involved in HRG-induced β-catenin phosphorylation and HER2/β-catenin association. A, MCF-7 cells were equilibrium-labeled with [32P]orthophosphate, pretreated with either MAP kinase inhibitor or PI 3-kinase inhibitors for 20 min, and then treated with HRG for 30 min. Cell lysates were immunoprecipitated with β-catenin antibody, analyzed by SDS-polyacrylamide gel electrophoresis, and autoradiographed. B, serum-starved MCF-7 cells were treated with HRG, and the cell lysates were immunoprecipitated (IP) with HER2 Ab and analyzed by immunoblotting (IB). The upper part of the blot was blotted with the indicated anti-phosphotyrosine PY69 Ab, followed by HER2 and finally by Tiam1 antibodies. The lower part of the same blot was blotted with β-catenin antibody and subsequently with anti-phosphotyrosine PY69 antibody. C, signaling pathways involved in HER2/β-catenin association. MCF-7 cells were pretreated with MAP kinase inhibitor PD98059, p38 MAP kinase inhibitor SB203580, and PI 3-kinase inhibitor LY294002 for 30 min, followed by 15 min of HRG treatment. HER2 was immunoprecipitated and Western blotted with β-catenin antibody.

Fig. 3. Role of PI 3-kinase on HRG-induced β-catenin nuclear signaling and ruffles formation. A, relative TCF-LEF1 promoter luciferase activation in MCF-7 cells co-transfected with different expression vectors blocking specific signaling pathways. After co-transfection, cells were treated with HRG for 12 h, where indicated. CON, control. B, confocal images of HRG-treated MCF-7 cells with or without pretreatment with signaling inhibitors and co-stained with TRITC-phalloidin (in red) and with a Tiam1 antibody (in green). PD, PD98059; LY, LY294002.
Tiam1 constructs. DNA staining is visualized for endogenous cells (\textsuperscript{b}) situated inside the cluster are red C1199-Tiam1- and C580-Tiam1-positive and if expressed by C1199-Tiam1 in both situations (\textsuperscript{b}).

We analyzed the transfected cells for subcellular localization of Tiam1 and \(\beta\)-catenin using confocal analysis, as described previously (38). Both C1199-Tiam1 and C580-Tiam1 constructs were as potent as the active \(\beta\)-catenin construct also presented for an easier visualization of the cellular junction aspect (\textit{D}). Confocal analyses of HA-tagged C1199-Tiam1- and C580-Tiam1-positive cells situated at the cell cluster periphery are costained for endogenous \(\beta\)-catenin (green) and each of the tagged Tiam1 constructs. Two different regions cropped from each of the left panels and shown by arrows are highly magnified and displayed in the right inserts for a more detailed visualization of the images presented merged in the left panels or separated for HA-Tiam1 (red) or \(\beta\)-catenin (green). Note the ruffles-inducing effect exerted by C1199-Tiam1 in both situations (\textit{C} and \textit{D}) as compared with less potent C580-Tiam1 constructs. DNA staining is visualized in blue in \textit{C} and \textit{D}.

Cells for Tiam1 and F-actin (Fig. 3B). Interestingly, HRG-induced ruffles formation was also PI 3-kinase-dependent (Fig. 3B).

\textbf{Tiam1 Overexpression Mimics HRG Effects on Cell Ruffles, Cell Cluster Decompaction, and Cell Migration}—To demonstrate that Tiam1 and \(\beta\)-catenin are downstream effectors of HRG-induced cell invasion (5) and scattering (10), we next asked whether overexpression of Tiam1 or \(\beta\)-catenin could mimic any of these effects. To explore this possibility, we cotransfected MCF-7 cells with the C1199-Tiam1 and C580-Tiam1 constructs or with the constitutively active \(\beta\)-catenin construct, together with a GFP-containing vector, to trace the transfected cells and perform an \textit{in vitro} cell invasion assay as described previously (38). Both C1199-Tiam1 and C580-Tiam1 contain intact catalytic and C-terminal pleckstrin homology (PH) domains but differ in their N-terminal domain (Fig. 4A) that is supposed to be responsible for membrane localization and ruffle formation in some cellular systems (33, 39–41). Our results showed that the N-terminal PH domain was able to significantly increase the invasive potential of MCF-7 cells as compared with cells transfected with C580-Tiam1 (Fig. 4B). Overexpression of the constitutively active \(\beta\)-catenin construct also promoted the invasive potential in the recipient cells (Fig. 4B).

These results showed that C1199-Tiam1 was more potent than C580-Tiam1 in increasing the migratory potential of MCF-7 cells. However, neither C580- nor C1199-Tiam1-overexpressed constructs were as potent as the active \(\beta\)-catenin mutant in increasing the invasive potential of MCF-7 cells. One possible explanation could be that overexpression of Tiam1 constructs remodeled the intercellular function stability and hence, triggered a series of downstream events leading to activation of the endogenous \(\beta\)-catenin signaling. Furthermore, the observed better effectiveness of the non-degradable \(\beta\)-catenin, as compared with either of the Tiam1 constructs, could also be related to a persistent activating effect on the transcription of pro-inflammatory genes. Interestingly, the C1199-Tiam1 and not the C580-Tiam1 appeared to disturb the cell to cell connections, even when overexpressed in cells that were localized inside the cell clusters, in addition to those situated at the periphery of cell clusters. Together, these spatial parameters may account for the difference in the invasion potential displayed by cells overexpressing the two Tiam1 constructs. Because Tiam1 was able to increase the migratory capacity of MCF-7 cells, we next asked whether Tiam1 overexpression can mimic the effects of HRG on cytoskeleton in MCF-7 cells. Using confocal analysis, we analyzed the transfected cells for subcellular localization of both the HA-tagged Tiam1 and the endogenous \(\beta\)-catenin (in the absence of the HRG signal) (Fig. 4, C and D). Because cells that overexpress the Tiam1 construct can be situated inside the cellular cluster and have strong intercellular connections with
the surrounding cells or that can be at the periphery of the cell cluster, with at least one cellular side free of intercellular connections, we analyzed both situations (Fig. 4, C and D). Positive cells (marked with a star) that overexpress the C1199-Tiam1 construct showed a membrane localization with a rather distorted intercellular interface as compared with C580-Tiam1-overexpressing cells, which displayed smooth intercellular connections (Fig. 4C, left panels). A closer examination of two different areas from the cell periphery (marked by arrows) allowed us to confirm the existence of loose intercellular connections between C1199-Tiam1-overexpressing cells and the neighbor cells, as compared with tight adhesive intercellular junctions in the case of C580-Tiam1-overexpressing cells (Fig. 4C, two right inserts, respectively). On the other hand, if the positive cell was situated at the periphery of the cellular cluster, C1199-Tiam1 overexpression was dramatically inducing ruffles formation on the whole cellular surface and at the cellular free edges, all characteristic features for a very motile phenotype. Moreover, both β-catenin and the HA-tagged C1199-Tiam1 were localized in the close proximity of these structures (Fig. 4D, upper panels). A similar effect could be seen in C580-Tiam1-overexpressing cells, which displayed a general tendency of cellular dissociation but with modest ruffles formation, where only a small amount of the HA-tagged Tiam1 could be seen in the proximity of the cellular membranes, most of it being intracytoplasmic (Fig. 4D, lower panels). These results demonstrate that C1199-Tiam1 by itself is a very potent modulator of the stability of the intercellular junctions and could mimic some of the HRG-induced motile features in MCF-7 breast tumor cells.

**Tiam1 Cooperates with HRG Signaling to Transactivate the LEF-1/β-Catenin Nuclear Signaling**—Because Tiam1 itself was able to disturb the intercellular adhesiveness by inducing cellular ruffles, as well as β-catenin redistribution to the highly dynamic cell membranes, we next investigated whether these effects of Tiam1 could influence the status of β-catenin-specific TCF-LEF1 nuclear signaling. First, we tested the effect of Tiam1 on LEF1 promoter activity in the presence or absence of HRG using Tiam1 constructs, C1199-Tiam1, and C580-Tiam1 (Fig. 5A). Overexpression of either Tiam1 construct significantly enhanced LEF1 promoter activity in the presence of HRG but only moderately, even if very consistently, in its absence (Fig. 5A). The observed transactivation of the LEF1-TCF promoter was mediated via β-catenin-LEF1-TCF complex formation, as there was no such transcriptional activation when cells were transfected with the LEF1-TCF promoter construct (34) containing mutations in the LEF1-TCF binding sites for β-catenin (Fig. 5A, hatched bars). Accordingly, introduction of a stabilized active β-catenin construct having defined mutations within the GSK3β-induced phosphorylation sites (42) was sufficient to maximally stimulate the promoter activity, with no further stimulatory effect of HRG (Fig. 5A).

Confocal analysis after transient transfection of MCF-7 cells with HA-tagged C1199-Tiam1 or C580-Tiam1 showed HA immunoreactivity at the cell membrane or at cellular cytoplasm, respectively, as described previously (Fig. 5C, left panels).
Tiam1-overexpressing MCF-7 cells showed a modest increase in the levels of specific dot-like LEF-1 nuclear immunoreactivity in response to HRG (Fig. 5B, left panels). Interestingly, HRG treatment of these cells resulted in significant nuclear HA immunoreactivity in cells overexpressing each of the two Tiam1 constructs (Fig. 5C, right panels). Together, these findings suggested that Tiam1 overexpression might potentiate HRG-induced β-catenin/LEF-TCF transcriptional activation and that dynamic cellular membranes containing Tiam1 and β-catenin proteins may be closely linked to its effect in the nuclear compartment.

**Tiam1 Overexpression Correlates with Mammary Tumor Progression**—To explore whether there is any potential correlation between Tiam1 and β-catenin expression levels and the invasiveness of human breast tumors, we analyzed a small number of breast cancer samples representing grade II and grade III characterized tumors (43). As shown in Fig. 6, grade III tumors had higher expression levels of Tiam1 (and of β-catenin, in some cases) compared with samples from grade II tumors, which may suggest that Tiam1 expression may be closely correlated with the invasive phenotypes of human breast tumors.

These results suggest that HRG may regulate the stability of intercellular junctions and that β-catenin tyrosine phosphorylation may be associated with alterations in its content inside the cytoplasm, on the dynamic cell membranes, or inside the nucleus. Because HER2 is a tyrosine kinase receptor, bound to the cellular membrane, one of the resulting hypotheses is that β-catenin situated at the level of cell membranes may serve as the initial target of the HRG-activated HER2 receptor. An increased cytoplasmic pool of β-catenin in HRG-stimulated mammary cancer epithelial cells may also facilitate its nuclear translocation. Dynamic modifications of these membranes upon HRG signaling, because of remodeling of the cell cytoskeleton, may further contribute toward the nuclear translocation of β-catenin.

Membrane targeting of cytoplasmic proteins and redistribution of protein complexes in distinct subcellular compartments represent two common characteristics of intracellular signaling. Upon stimulation with growth factors, cells undergo reorganization of the cytoskeleton and rapid recruitment of a multitude of signaling molecules to the receptors (1, 2, 44). For example, Sox-1, a GEF for Ras, translocates upon stimulation of Rat1 cells with epidermal growth factor (45–47). Similarly, Tiam1, a GEF for Rac1, associates with the membrane fraction upon growth factor stimulation, and this association is required for membrane ruffling and c-Jun N-terminal kinase activation (39). Nucleotide exchange factors that act on Rho proteins contain two key conserved domains, a DbI homology domain, which is believed to be responsible for catalyzing GDP/GTP exchange, and a PH domain, which seems to be important for cellular localization through interaction with lipids or protein. Some GEFs such as Tiam1 carry a second PH domain situated at the N-terminal and mediate Tiam1 localization at the cell membrane and ruffles (39, 48–50). Furthermore, phospholipids may also play an important role in determining the cellular localization of Tiam1, as both phosphatidylinositol-2 and phosphatidylinositol-3 phosphates bind to its N-terminal PH domain, and Tiam1 activation of Rac1 requires PI 3-kinase activity (40).

The data presented here demonstrate that HRG stimulates the relocation of Tiam1 to the membrane fractions with consequent intercellular dissolution, and our results suggest that Tiam1 relocalization may influence cellular invasiveness. The membrane redistribution and phosphorylation of Tiam1 occur rapidly enough to be involved in this activation. Previously, another growth factor, platelet-derived growth factor, was shown to induce phosphorylation of Tiam1 in Swiss 3T3 fibroblasts, and calmodulin K II mediated its translocation to the membranes (49, 51). Through the use of pharmacological inhibitors, we have now shown the predominant involvement of PI 3-kinase in the translocation of Tiam1 to the membrane in HRG-stimulated breast cancer cells.

Cell-cell junctional proteins are a common target of mitogenic factors initiating cell migration (52). In this context, HRG, a known invasive factor for MCF-7 cells, induces a rapid phosphorylation of β-catenin and Tiam1. These effects were associated with a perturbation of the E-cadherin-mediated cell-cell adhesion function and preceded both the induction of cell migration and β-catenin nuclear translocation and transcription of the LEF-TCF-β-catenin reporter. Cellular membranes, such as active ruffles, or leading cellular edges seemed to be the point at which Tiam1 or HRG may affect β-catenin nuclear signaling. At least three distinct steps may be considered. The first step may be at the level of the cell membrane, the second may be at the level of the cytoplasm, and the third may be inside the nucleus. Several HRG-induced signals could affect β-catenin-regulated activation of the LEF-TCF-β pathway. One pathway that seemed to control each of these steps was activation of PI 3-kinase, as suppression of this pathway resulted in a blockage of the HER2/β-catenin association, cytoplasmic accumulation of phosphorylated β-catenin, or its nuclear translocation and activation of LEF-TCF promoter activity. Alternatively, it is also possible that HRG may use an additional mechanism of increasing the cytoplasmic pool of β-catenin, by blocking its degradation through AKT activation, which is known to dephosphorylate and inactivate GSK3β (data not shown). The role of Tiam1 during these events is supported by two main lines of evidence, first by its binding to the dynamically active cell membranes and its phosphorylation after HRG stimulation and second by inducing cellular ruffles, increasing the migratory behavior of the cell, and augmenting the HRG-induced LEF-TCF-β-catenin activation after its overexpression. Future studies exploring the mechanism by which Tiam1 affects nuclear transactivation are likely to provide additional insights about the role of Tiam1 in breast cancer invasion.

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