Heregulin Regulates Cytoskeletal Reorganization and Cell Migration through the p21-activated Kinase-1 via Phosphatidylinositol-3 Kinase*

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The mechanisms through which heregulin (HRG) regulates the activities of breast cancer cells are currently unknown. We demonstrate that HRG stimulation of non-invasive breast cancer cells enhanced the conversion of globular to filamentous actin and the formation of membrane ruffles, stress fibers, filopodia, and lamellipodia and accompanied by increased cell migration. In addition, HRG triggered a rapid stimulation of p21-activated kinase1 (PAK1) activity and its redistribution into the leading edges of motile cells. The HRG-induced stimulation of PAK1 kinase activity followed phosphatidylinositol-3 kinase (PI-3 kinase) activation. Inhibition of PI-3 kinase activity blocked the activation of PAK1 kinase and also blocked cell migration in response to HRG. Furthermore, direct inhibition of PAK1 functions by the dominant-negative mutant suppressed the capacity of HRG to reorganize actin cytoskeleton structures. We also demonstrated that HRG stimulation promoted physical interactions between PAK1, actin, and human epidermal growth factor receptor 2 (HER2) receptors, and these interactions were dependent on the activation of PI-3 kinase. The blockade of HER2 receptor by an anti-HER2 monoclonal antibody resulted in the inhibition of HRG-mediated stimulation of PI-3 kinase/PAK pathway and also the formation of motile actin cytoskeleton structures but not extracellular signal-regulated kinases. These findings suggest a role of PI-3 kinase/PAK-dependent reorganization of the cortical actin cytoskeleton in HRG-mediated increased cell migration, and these changes may have significant consequences leading to enhanced invasion by breast cancer cells.

Proto-oncogenes are a group of normal genes that play important roles in the regulation of cell proliferation, differentiation, and viability. Abnormalities in the expression, structure, or activity of proto-oncogene products contribute to the development and maintenance of the malignant phenotype. For example, HER2 (also known as c-erbB2 or c-neu) encodes a 185-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity (1) that has been shown to be overexpressed, amplified, or both, in a number of human malignancies, including breast cancer (2). Overexpression of the HER2 receptor is associated with increased progression and metastasis, an aggressive clinical course, and decreased disease-free survival in human breast cancer (3, 4). Recently, two additional members, HER3 and HER4, have been added to the human EGF receptor (HER) family. All of these receptors share sequence homology with the tyrosine kinase domain of HER1 (5). Overexpression of some growth factor receptors has been shown to induce transformed properties in recipient cells (6), possibly because of excessive activation of signal transduction pathways. The regulation of HER family members is complex, as they can be transactivated by heterodimeric interaction between two HER members and thus can utilize multiple pathways to execute their biological functions (7, 8). For example, HER3 and HER4 receptors bind to more than a dozen isoforms of the heregulins (HRGs) or neu differentiation factors (9, 10), and they can activate the HER2 receptor as a result of HER2/HER3 or HER2/HER4 heterodimeric interactions (7, 8). A ligand that interacts with HER2 in the absence of other HER family members has yet to be identified.

Although the significance of HER2 in breast cancer is well established, the mechanism involved remains poorly understood. It has been proposed that this may involve constitutive activation of the intrinsic tyrosine kinase activity due to either mutations in the HER2 gene, overexpression, and/or transactivation via receptor-dimerization with other HER members. HER3 is unique among HER family members, as it has an impaired tyrosine kinase domain due to substitution of three amino acids in the kinase domain (11). Despite the kinase dead nature of HER3, HRG binding to HER3 leads to increased HER3 tyrosine phosphorylation, probably due to formation of a high-affinity co-receptor complex through heterodimeric interaction with HER2 (12). It is believed that among the HER family, the HER2/HER3 dimer complex elicits the most potent mitogenic signal (7). This may be related to the fact that the COOH-terminal phosphorylation domain of HER3 contains several consensus sites for the binding of signal-transducing proteins implicated in mitogenic signaling, including phosphatidylinositol-3-kinase (PI-3 kinase). HRG stimulation of breast cancer cells enhances activation of PI-3 kinase and MAP-kinases (13, 14). Despite the widely acknowledged role of the HER2/herregulin pathway in breast cancer progression, the signaling pathway(s) involved remains elusive.

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The abbreviations used are: HRG, heregulin-β1; PAK, p21-activated kinase; MBP, myelin basic protein; PI-3 kinase, phosphatidylinositol-3 kinase, HA, hemagglutinin epitope; Ab, antibody; mAb, monoclonal Ab; EGF, epidermal growth factor; HER, human EGF receptor; MMP, matrix metalloproteinase.
The exposure of cells to growth factors has been shown to cause cytoskeleton reorganization, formation of lamellipodia, membrane ruffling, and altered cell morphology, and accordingly, it has been implicated in stimulating cell migration and invasion (15, 16). Most eukaryotic cells possess the capacity to move over or through a substrate, and cell migration plays a key role in both normal physiology and diseases, including invasion and metastasis (17). In many tissues, the motility function of cells is normally repressed but can be activated by appropriate stimuli and/or oncogenic transformation. In fact, one of the earliest response of cells to many extracellular growth factors is rapid reorganization of their cytoskeleton and cell shape. The leading edge of a motile cell is composed of thin protrusions of membrane that continuously extend and retract, mediating the initial stage of cell movement and determining the direction of advance. The underlying cytoskeleton of a leading edge is composed of actin-filament bundles (in filopodia) or meshworks (in lamellipodia) oriented toward the membrane (16, 17). In addition, the cell motility also involves the formation of long bundles of actin stress fibers that end in focal adhesions, points of attachment of the plasma membranes to the substratum.

The small GTPases, including Cdc42, Rac1, and RhoA, have been implicated in the regulation of morphology; the formation of filopodia/lamellipodia, membrane ruffles, and stress fibers; and motility of mammalian cells (15). The small GTPase shuttles between the active GTP-bound form and the inactive GDP-bound form and act as molecular switches in intracellular signaling. There is also evidence to suggest that the small GTPases are effectors of PI-3 kinase in the signal transduction pathway leading to growth factor-induced actin reorganization, membrane ruffling, and chemotaxis (18–20). Although the small GTPase family members have been shown to be necessary for morphological changes in response to growth factors, the mechanisms by which they initiate and regulate the formation of cytoskeletal structures are not completely understood.

Recently, a family of serine/threonine kinases known as p21-activated kinases (PAKs) have been identified as a direct target of activated GTPases (21, 22). Activation of PAK1 has been shown to result in phenotypic changes reminiscent of those produced by GTPases. The activation of PAK1 either by overexpression of dominant active PAK1 mutants or by stimulation of cells with growth factors causes the accumulation of F-actin and the formation of membrane ruffles, lamellipodia, and filopodia. Furthermore, activated PAK1 has been shown to be co-localized with F-actin at the leading edge of cells (21, 22).

Growth factors play an essential role in the regulation of proliferation of mammary epithelial cells. HRG has been shown to be involved in morphogenesis and ductal migration in mammary epithelium (23, 24). In addition to HER2 overexpression, accumulating evidence suggests that the HRG pathway may be involved in the progression of breast cancer cells to a more invasive phenotype. In the present study, we investigated the mechanism(s) by which HRG-p1 signaling may participate in the acquisition of invasive phenotype in breast cancer. Our results demonstrate that HRG stimulation of a noninvasive human breast cancer cell leads to the activation of PAK1 kinase via PI-3 kinase; an increased physical association between the activated PAK1, actin, and HER2 receptor; and a redistribution of PAK1 at the leading edges. These interactions were dependent on the activation of PI-3 kinase by HRG. In addition, these molecular changes were accompanied by the development of lamellipodia/filopodia and stress fibers, and they also increased the migration of breast cancer cells through a porous membrane.
cells trans-migrating to the lower side of the membrane (facing the lower chamber) and expressed as percentage of the total cells. The results shown in Fig. 1c demonstrate that HRG is a very potent cell migratory growth factor.

Because overexpression of HER2 has been shown to be frequently associated with an aggressive clinical course in human breast cancer (4), we also examined the effect of HER2 overexpression on the cell migration and reorganization of actin-containing structures, using well characterized HER2-overexpressing (MCF-7/HER2) cells (25, 26). Results demonstrated that HER2-overexpressing MCF-7 cells were more motile and have more constitutively polymerized actin at the free edges as compared with MCF-7 cells but responded to HRG by further enhanced actin polymerization and cell migration (data not shown).

**HRG Stimulates PAK1 Activation and Cell Migration**—Because activation of PAK1, the downstream target of GTPases, has been shown to result in the phenotypic changes, including a formation of lamellipodia and filopodia (21, 22), we explored the potential involvement of PAK1 in the action of HRG. To evaluate this possibility, we first investigated whether PAK1 is activated by HRG in MCF-7 cells. Cell lysates from control and HRG-treated (30 min) MCF-7 cells were immunoprecipitated with anti-PAK1 Ab, and precipitated PAK1 was assayed for its kinase activity using exogenous MBP as substrate. The results shown in Fig. 2d demonstrate that HRG rapidly increases PAK1 kinase activity, and there was autophosphorylation of PAK1. In addition to activating PAK1 kinase, the activation of PI-3 kinase has also been shown to induce actin reorganization and membrane ruffling (18), possibly through guanine exchange factors. Because HRG is known to activate PI-3 kinase in breast cancer cells, we investigated the potential role of PI-3 kinase in HRG-mediated alterations in cytoskeletal structures.

To this end, we first examined the kinetic relationship between the stimulation of HER2 and PI-3 kinase and PAK1 activities. As shown in Fig. 2b, c, d, activation of HER2 receptors by binding of HRG to its receptors resulted in rapid increase in the activities of all three kinases. The PI-3 kinase activity was induced to a maximum 13.3-fold compared with control cells within 5 min of HRG treatment and persisted up to 5 h post-treatment. It was noteworthy that HRG-induced activation of PAK1 followed the kinetics of PI-3 kinase activation with a maximum 7-fold activation by 30 min of treatment, and PAK-1 kinase remained activated over the period of 5 h compared to the levels in control untreated cells.

Because HRG-induced activation of PAK1 followed the kinetics of PI-3 kinase, we investigated whether PAK1 activation is a downstream event of HRG-induced activation of PI-3 kinase. The results shown in Fig. 2e–g, demonstrate that inhibition of PI-3 kinase by a specific inhibitor, wortmannin, was accompanied by concurrent inhibition of HRG-induced activation of PAK1 in MCF-7 cells (Fig. 2, e and f). Interestingly, treatment of MCF-7 cells with wortmannin was also accompanied by suppression of HRG-mediated stimulation of cell migration through a porous membrane (Fig. 2g). The results shown in Fig. 2e also demonstrate that although wortmannin partially inhibited the production of phosphatidylinositol 3-phosphate, it completely blocked both PAK1 activity and cell migration in HRG-treated cells. It is possible that a phosphotyrosine immunoprecipitation that was used to measure PI-3 kinase activity also contains other PI kinases that are not sensitive to wortmannin.

The results in Fig. 3 show the redistribution of PAK1 at the motile free edges in the polarized, migrating HRG-treated cells. In HRG-activated cells, PAK-1 was clearly colocalized to characteristic peripheral complexes situated internal from the cell border but in the proximity of the area that resembles the leading edge of a cell that becomes polarized due to cell movement. A cytoplasmic punctate distribution could also be seen around the cell nucleus. Control cells consistently displayed different distribution of PAK-1, where it colocalized mainly with cytoskeleton. Different subcellular localizations, such as focal adhesion complexes or pinocytic vesicles of exogenously introduced PAK-1, and not its endogenous activation as we describe here, have been stated before (22). Indeed, PAK-1 subcellular localization have been shown to depend on its expression level and on its interplay with GTPases (21, 22).

**Dominant-negative PI-3 Kinase Mutant and a PAK1 Mutant Prevent HRG-mediated Reorganization of Actin-containing Structures—Actin reorganization is the central molecular
event essential for the development of motile cytoskeletal structures in response to an appropriate stimulus. In order to establish that inhibition of cell migration by wortmannin was mediated by suppression of PI-3 kinase, we also inhibited PI-3 kinase function by the direct expression of a HA-tagged dominant-negative human mutant p85\(\alpha\) (DNp85), which lacks a binding site for p110 (32), and determined the effect on HRG-mediated reorganization of actin in MCF-7 cells. As demonstrated in Fig. 4, a–c, inhibition of PI-3 kinase in transfected HA-positive cells (green color, arrow) resulted in suppression of HRG-mediated actin polymerization. In contrast, active actin polymerization (stress fibers) was observed in cells that did not express the mutant PI-3 kinase (negative cells, arrowhead). The inhibitory effect of DNp85 on actin structures was observed in more than 50% of the transfected cells. The observed suppression of actin polymerization by DNp85 was HRG-dependent, as there was no effect of overexpression of ΔNp85 alone (data not shown).
In order to demonstrate that PAK-1 is downstream of PI-3 kinase in HRG signaling and is involved in mediating the effects of HRG on cytoskeletal system, we examined the effect of direct expression of a Myc-tagged PAK-1 mutant on HRG-induced alterations in the reorganization of actin in MCF-7 cells. A mutated PAK1 molecule (DH83L/H86L) chosen for these studies contains mutations within the p21 (Rac/Cdc42) binding site, rendering it unable to associate with Rac and Cdc42 (21). Although active as a protein kinase, this form of Pak1 may sequester downstream signaling elements, thus potentially interfering with Rac and/or Cdc42 signals. The results shown in Fig. 4, d–f, demonstrate that inhibition of PAK1 function in cells transfected with the Myc-tagged dominant active PAK1 mutant (green color, arrow) resulted in complete blockade of HRG-mediated actin polymerization. In transfected cells, F-actin was weakly seen at cell-cell contact sites, but it was not highly polymerized. In contrast, actin was highly polymerized in the untransfected cells (arrowhead). The inhibitory effect of dominant-negative PAK1 on actin structures was observed in more than 60% of the transfected cells. There was no inhibitory effect of the transfection of Myc-tagged control vector on HRG-induced reorganization of actin polymerization or cell attachment (Fig. 4, g–i). In brief, these results suggested that the HRG-initiated signals leading to actin reorganization could be blocked by inhibiting PI-3 kinase and/or PAK1 kinase.

HRG Enhances Physical Interaction between HER2, PAK1, and Actin—Because HRG triggered relocalization of PAK1 into the leading edge of cells, we explored the potential interactions between the activated HER2 receptor and the PAK1 and actin by immunoblotting PAK1 immunoprecipitates with an anti-HER2 mAb. A modest but significant amount of HER2 receptor was associated with PAK1/actin complexes only in HRG-stimulated MCF-7 cells (Fig. 5b, lanes 1 and 2). As a control, cell lysates from HRG-treated or control MCF-7 cells were immunoprecipitated with normal rabbit serum, and there was no co-immunoprecipitation of actin or HER2 or PAK1 (Fig. 5b, lanes 3 and 4). The observed interactions among HER2, PAK1, and actin were dependent on the activation of PI-3 kinase by HRG, as pretreatment of MCF-7 cells with the PI-3 kinase inhibitor wortmannin disrupted the interaction between PAK1 and HER2 receptor and actin (Fig. 5b). The results shown in Fig. 5b also demonstrate that there was detectable interaction between the HER2 receptor and PAK1 in HER2-overexpressing MCF-7 cells, and HRG stimulation could further enhance
the interactions among HER2 receptor, PAK1, and actin. These results are consistent with the existence of motile cytoskeleton structures in MCF-7/HER2 cells. Results of other experiments indicated that HER2-overexpressing MCF-7 cells have 2–3 times more baseline PAK1 activity than MCF-7 cells (data not shown). These results suggest that HRG stimulates physical interactions among HER2, PAK1, and actin. It remains to be investigated whether these interactions are direct or mediated through other protein(s).

An Essential Role of HER2 Receptor in HRG-mediated Actin Reorganization and Increased Cell Migration—To investigate the possible contribution of HER2 receptors in HRG-induced motile responses in breast cancer cells, we examined the effect of an anti-HER2 mAb, 4D5 (27), on the development of motile structures by HRG in an extracellular matrix, Matrigel. The results shown in Fig. 6a demonstrate that treatment of MCF-7 cells with mAb 4D5 was accompanied by complete suppression of HRG-mediated development of F-actin containing structures by HRG. The ability of anti-HER2 mAb 4D5 to suppress the cell migratory function of breast cancer cells was also verified by the Boyden chamber assay. The results shown in Fig. 6b demonstrate that co-treatment of MCF-7 cells with mAb 4D5 and HRG resulted in the suppression of HRG-induced cell migration. Because one of the most important steps during the process of migration and/or invasion is the destruction of extracellular matrix by matrix metalloproteinases (MMPs) (34), we examined the potential regulation of MMPs by HRG and its blockage by mAb 4D5. The results in Fig. 6c show that stimulation of MCF-7 cells with HRG leads to a significant increase in the expression of MMP-9 protein, as well as its gelatinolytic activity in the conditioned medium, and these changes could be prevented by co-treatment with mAb 4D5.

Anti-HER2 mAb 4D5 Blocks HRG-induced Signaling Pathways and Interaction between HER2 and HER3 Receptors—To further delineate the mechanism of inhibitory effects of mAb 4D5 on HRG-mediated functions in MCF-7 cells, we examined the effect of blocking HER2 receptor by mAb 4D5 on HRG-activated signaling pathways. MCF-7 cells were pretreated with or without mAb 4D5 or an unrelated anti-EGF receptor mAb 225 for 30 min, followed by stimulation with HRG for 30 min, and cell lysates were assayed for the activation of HER2, PI-3k, PAK1, JNK, and ERK kinases. As shown in Fig. 7a, co-treatment of cells with mAb 4D5 failed to block the activation of HER2 receptor by HRG and, in contrast, resulted in the activation of HER2 receptor (top panel, lane 3). However, mAb 4D5 treatment was accompanied by 67% suppression in the levels of HRG-induced PI-3 kinase activity. Treatment with mAb 225 blocked the HRG-mediated activation of PI-3 kinase by 25.7%. Treatment with mAb 4D5 also resulted in the inhibition of HRG-induced activation of PAK1 kinase and JNK kinase activities to a significant extent (Fig. 7b). In contrast, it was interesting to note that mAb 4D5 failed to block the activation of ERK kinase by HRG (Fig. 7b, bottom panel). The results shown in Fig. 7a also demonstrate that mAb 4D5-induced the activation of HER2 receptor was not accompanied by activation of any signaling kinases analyzed in this study, thus suggesting the possibility of a nonproductive nature of HER2 activation by mAb 4D5 in MCF-7 cells. In the past, mAb 4D5 has been shown to induce the activation of HER2 pathway in breast cancer cells overexpressing HER2 receptors (35), but not in cells with a normal level of HER2 receptors, such as MCF-7 cells, as shown in the present study. Taken together, the above findings suggested that mAb 4D5 may selectively inhibit the activation of PI-3 kinase-dependent pathway leading to actin reorganization, but not the signals leading to ERK2 activation.

To further understand the biochemical basis of inhibitory effects of mAb 4D5 in MCF-7 cells, we explored the possibility of an interfering effect of pretreatment of mAb 4D5 on HRG-induced interactions between HER2 and HER3 receptors. The results shown in Fig. 7c demonstrate that pretreatment of cells mAb 4D5 for 30 min blocked interactions between HER2 and HER3 receptors in HRG-treated cells, because there was no co-immunoprecipitation of HER3 receptor with HER2 mAb in cells cotreated with mAb 4D5 and HRG (lane 4) compared with the cells treated with HRG alone (lane 2) or mAb 225 and HRG together (lane 6). As shown in Fig. 7c, there was no down-regulation of HER2 receptor by 1 h of treatment with mAb 4D5.

DISCUSSION

Abnormality in the action of the HER2 receptor and HRG, which activates HER2 receptor via its binding to HER3 or HER4 receptors, has been shown to be frequently associated with the progression of breast cancer cells to a more invasive and aggressive phenotype. Because the process of progression to a more malignant phenotype must involve physical locomotion of cancer cells, and because HRG has been shown to influence ductal migration in mouse mammary epithelium (23, 24), we investigated the nature of HRG-generated signals that may be involved in the regulation of cell migration. The results presented here indicate that HRG treatment of noninvasive
MCF-7 breast cancer cells leads to a significant enhancement of cell migration. Our conclusion that HRG is a very active cell migratory factor is supported by the following lines of evidence: (i) HRG-mediated increased expression of F-actin was accompanied by the development of motile structures, such as filopodia and lamellipodia; (ii) HRG stimulated the formation of stress fibers, the appearance of a leading edge, and membrane ruffles at the leading edge; (iii) HRG stimulated the transmigration of MCF-7 cells across an 8-μm porous membrane; and (iv) HRG also induced cell spreading and F-actin containing structures when cells were cultured on a reconstituted basal membrane, Matrigel. Taken together, these data provide evidence that HRG induces the formation of motile actin cytoskeleton structures and is a very potent migratory factor for breast cancer cells.

Among the various pathways leading to actin reorganization, the Rho family of the small GTPases, including cdc42, Rac1, and RhoA, has been implicated in the regulation of morphology and motility of mammalian cells. Recent studies have identified the p21-activated kinases (PAKs) as a direct target of HRG.
activated GTPases, because activation of PAK1 has been shown to result in the phenotypic changes reminiscent of those produced by Rac and/or cdc42 (21, 22). Furthermore, a direct role of PAK in promoting cell migration has been suggested by the physical interaction between PAK1 and F-actin in response to upstream stimuli (21, 22). In this context, our observation that HRG rapidly stimulated PAK1 kinase activity and also its relocalization to the leading edges and membrane ruffles of the activated cells opens a new area of investigation linking, for the first time, HER2 activation to cytoskeleton signaling in breast cancer cells. The involvement of PAK1 activation in HRG signaling was evident from the following independent findings: (i) PAK1 activation followed the kinetic tyrosine phosphorylation of HER2 (Fig. 2); (ii) activated PAK1 activity has been shown to be associated with receptor tyrosine kinases (36); and (iii) PAK1 physically associated with the HER2 receptors only in HRG-stimulated cells and not in control cells (Fig. 5). The significance of PAK1 activation in the action of HRG upon cell motility was deduced from the observations that HRG triggered a relocalization of PAK1 to the leading edges of activated cells, the PAK1 physically associated with actin in HRG-stimulated cells, and the expression of a PAK1 mutant completely prevented HRG-mediated actin polymerization required for the reorganization of actin-containing structures. These findings suggest a role of PAK1 activation in HRG-mediated reorganization of actin-containing motile structures in breast cancer cells.

Data from the literature (18) and from these studies suggest that the activation of PI-3 kinase is a common growth factor response that may be involved in actin reorganization and membrane ruffling. The finding that the stimulation of PAK1 kinase activity follows PI-3 kinase activation is important, as it implies that PI-3 kinase may constitute an initial HRG-activated signal for actin reorganization and cell migration. This view is further supported by the observations that (i) inhibition of PI-3 kinase by the specific inhibitor wortmannin was accompanied by concurrent inhibition of HRG-mediated stimulation of PAK1 activity and cell migration (Fig. 2, e–g); (ii) expression of dominant-negative PI-3 kinase or PAK-1 mutant blocked HRG-mediated reorganization of actin-containing structures (Fig. 4); (iii) inhibition of PI-3 kinase activity by wortmannin completely prevented physical interactions between PAK1 and HER2 receptor and actin in HRG-treated cells (Fig. 5b); and (iv) anti-HER2 mAb 4D5 inhibited both activation of PI-3 kinase/PAK pathway and HRG-induced cell migration (Fig. 7). These results suggest that PAK1 may be downstream of PI-3 kinase in the HRG signaling pathway, leading to cytoskeletal reorganization and increased migration of breast cancer cells.

Another notable finding in this study was the essential role of HER2 receptor in mediating the cell migratory function of HRG. The evidence that a growth inhibitory anti-HER2-mAb 4D5 has the capacity to inhibit the biological effects of HRG, including reorganization of actin cytoskeleton and migration of breast cancer cells, is of special interest, as it strongly suggests that HRG primarily utilizes the HER2 receptor to transduce its signals in breast cancer cells. This hypothesis is further supported by our findings that pretreatment of MCF-7 cells with anti-HER2 mAb 4D5, and not an unrelated anti-EGF receptor mAb 225, completely blocked the development of motile actin-containing structures. At the moment, we do not know the mechanism by which anti-HER2 mAb 4D5 executes its pro-migration function. It is possible that binding of anti-HER2 mAb to HER2 receptors may lead to a conformational change in the HER2 receptor in a manner that interferes with its interaction with HER3 or HER4 receptors in response to HRG. Alternately, it is also possible that mAb 4D5 binding to HER2 receptor may create steric hindrance so that HER2 cannot efficiently interact with HER3 receptor, and/or pretreatment with mAb 4D5 may reduce the levels of HER2 monomers available for interaction with HER3 in response to HRG. In either event, mAb 4D5 treatment may result in the possible blockade of signals, initiated by the formation of HER2/HER3 heterodimers in HRG-treated cells. At the moment, we do not understand why mAbs blocked the activation of the PI3 kinase-PAK pathway but not that of ERK2. Because there is a PI-3 kinase binding motif in the carboxyl-terminal domain of HER3 receptors (37), one simple explanation may include direct recruitment of PI-3 kinase by activated HER3 receptor and ERK2 kinase may be activated by HER2. In this context, it is possible that the observed activation of HER2 receptor by mAb 4D5 could be due to the formation of HER2/HER2 homodimers. Studies are in progress to investigate these and other possibilities. Regardless of the mechanism, our findings have clearly established that anti-HER2 mAb 4D5 inhibits the development of actin-based motile structures as well as cell migration.

In the present study, we have not delineated the pathway(s) that connects the PI-3 kinase with PAK1 kinase in HRG-stimulated cells. Previous studies have shown that the small GTPases Rac1 and cdc42 are both activators of PAK1 kinase and effectors of PI-3 kinase in the signaling pathways. Ongoing studies are aimed at defining the roles of phosphorylated inositides and the small GTPases in HRG signaling. In this context, it was interesting to note that the expression of a dominant-negative PAK1 not only blocked the expected reorganization of actin at the leading edges but also blocked formation of stress fibers (Fig. 4, d–f). In the past, stress fiber formation has been shown to require RhoA activation and tyrosine phosphorylation (38). Therefore, it is possible that RhoA may be in the pathway(s) leading to PAK1 activation in HRG-treated cells. Alternatively, RhoA may be downstream or distantly regulated by PAK1 in HRG signaling. In either event, inhibition of PAK1 activity may lead to suppression of RhoA-dependent formation of stress fibers, therefore indicating a role for RhoA in HRG signaling. This is further supported by our recent finding that the inactivation of endogenous Rho by its specific ADP-ribosylation using botulium C3 exoenzyme blocks HRG-mediated formation of stress fibers. Taken together, our present findings of regulation of actin reorganization and cell migration functions by HRG and its complete suppression by an anti-HER2 mAb open a new avenue of investigation closely linking HER2 receptor, cytoskeleton signaling, and breast cancer cell activity. In addition, our present finding raises the possibility of therapeutic effects of mAb 4D5 in inhibiting the invasion and metastasis of breast cancer.

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