RAFTK/Pyk2 tyrosine kinase mediates the association of p190 RhoGAP with RasGAP and is involved in breast cancer cell invasion

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Focal adhesions and actin cytoskeleton are involved in cell growth, shape and movement and in tumor invasion. Mitogen-induced changes in actin cytoskeleton are accompanied by changes in the tyrosine phosphorylation of several focal adhesion proteins. In this study, we have investigated the role of RAFTK, a cytoplasmic tyrosine kinase related to focal adhesion kinase (FAK), in heregulin-mediated signal transduction in breast cancer cells. Stimulation of T47D cells with heregulin (HRG) induced the tyrosine phosphorylation of RAFTK and the formation of a multiprotein complex. Analyses of the members of the HRG-stimulated complex revealed that RAFTK is associated with p190 RhoGAP (p190), RasGAP and ErbB-2, and plays an essential role in mediating the tyrosine phosphorylation of p190 by Src. Mutation of the Src binding site within RAFTK (402) abolished the phosphorylation of p190. In addition, upon HRG stimulation of T47D cells, association of ErbB-2 with RAFTK was observed and found to be indirect and mediated by Src. Expression of wild-type RAFTK (WT) significantly increased MDA-MB-435 and MCF-7 breast cancer cell invasion, while expression of the kinase-mutated RAFTK-R457 (KM) or the Src binding site mutant RAFTK (402) did not affect this cell invasion. Furthermore, HRG leads to the activation of MAP kinase which is mediated by RAFTK. These findings indicate that RAFTK serves as a mediator and an integration point between the GAP proteins and HRG-mediated signaling in breast cancer cells, and implicate RAFTK involvement in the MAP kinase pathway and in breast cancer cell invasion. *Oncogene* (2000) 19, 1318–1328.

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Introduction

A variety of human tumors overexpress the ErbB (HER) family of type I receptor protein tyrosine kinases (Holmes *et al.*, 1992; Slamon *et al.*, 1989). Four members of this family are presently known: p170^{ErbB-1} (EGF-R), p185^{ErbB-2}, p180^{ErbB-3} and p180^{ErbB-4} (Bandyopadhyay *et al.*, 1998). In particular, the overexpression of the p185^{ErbB-2} has been repeatedly

implicated in human breast cancer and correlated with a poor clinical prognosis (Beerli *et al.*, 1995).

Heregulin (HRG) belongs to a subfamily of neu-regulins which are epidermal growth factor (EGF)-like ligands that bind and activate both ErbB-3 and ErbB-4 (Cohen *et al.*, 1996). HRG can activate the ErbB-2 receptor as a result of ErbB-2/ErbB-3 or ErbB-2/ErbB-4 heterodimeric interactions (Karunagaran *et al.*, 1996). HRG can either elicit a growth arrest and differentiation phenotype resulting in morphological changes, induction of milk component synthesis (casein and lipids) (Kumar *et al.*, 1996), and expression of intracellular adhesion molecule-1, or induce a mitogenic response, depending on the cell type studied (Sepp-Lorenzino *et al.*, 1996). HRG stimulation of non-invasive breast cancer cells enhanced the conversion of globular actin to filamentous actin and the formation of membrane ruffles, stress fibers, filopodia, and lamellipodia, and was also accompanied by increased cell migration (Adam *et al.*, 1998). Regulation of cytoskeleton reorganization and cell migration by HRG were found to be mediated by the p21-activated kinase-1 via phosphatidylinositol-3 kinase (Adam *et al.*, 1998). However, breast cancer cell invasion upon HRG stimulation is not well defined.

Members of the small GTPase superfamily have emerged as key regulators of the actin cytoskeleton (Hall, 1998; Ridley *et al.*, 1992); and the actin cytoskeleton has been implicated in many cellular functions, including motility, chemotaxis, cell proliferation, differentiation, endocytosis, secretion and cell polarization (Keely *et al.*, 1997; Ridley and Hall, 1992; Ridley *et al.*, 1992; Nobes *et al.*, 1995). In addition, Rho family GTPases play an important role in the activation of stress response pathways leading to the generation of pro-inflammatory mediators and/or apoptotic cell death. Rho GTPases have also been implicated in the regulation of transcriptional events that contribute to cell growth regulation (Keely *et al.*, 1997). Recently, Rac 1 and CDC42 have been implicated in the polarization of mammary epithelial cells and the promotion of their motility and invasion (Keely *et al.*, 1997).

Small GTPases are regulated by GAPs (GTPase-activating proteins), GEFs (GDP-GTP exchange factors) and GDIs (GDP dissociation inhibitors) (Machesky and Hall, 1996). The RhoGAP molecules, which preferentially recognize the GTP-bound form of the Rho proteins, stimulate their intrinsic GTPase activity and may therefore function as negative regulators (Li *et al.*, 1997). In transformed or growth

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factor-stimulated cells, p190 RhoGAP (p190) becomes tyrosine-phosphorylated and forms a distinct complex with RasGAP and the phosphorylated cytoplasmic protein, p62 dok (McGlade *et al.*, 1993; Ridley *et al.*, 1993). This complex may serve to coordinate Ras- and Rho-mediated signaling pathways (Settleman *et al.*, 1992a,b). Phosphorylation of p190 by Src, Abl or Blk *in vivo* at both tyrosine residues 1087 and 1105 was shown to be essential for the association of p190 to RasGAP (Hu and Settleman, 1997; Nakahara *et al.*, 1998). In addition, c-Src regulates the simultaneous rearrangement of actin cytoskeleton and the association of p190 and RasGAP following EGF stimulation (Chang *et al.*, 1995).

The mitogen-induced changes in the actin cytoskeleton are accompanied by dramatic changes in the tyrosine phosphorylation of several proteins present in focal adhesions such as vinculin, paxillin, focal adhesion kinase (FAK), p130cas, talin and tensin (Ojaniemi and Vuori, 1997). Focal adhesions are regions where indirect contact of cells with the extracellular matrix takes place. They provide anchorage sites for actin stress fibers and form a link between the extracellular matrix and the actin cytoskeleton via the integrin family of cell receptors (Jockusch *et al.*, 1995).

We have cloned and characterized a novel human cytoplasmic tyrosine kinase termed RAFTK (for Related Adhesion Focal Tyrosine Kinase) (Avraham *et al.*, 1995), also known as Pyk2, CAK- β or CADTK (Lev *et al.*, 1995; Sasaki *et al.*, 1995). RAFTK is related to FAK (48% identity, 65% similarity). Association of RAFTK with different protein tyrosine kinases (PTKs) such as Src, Fyn, and Csk as well as with the adaptor protein Grb2 was reported (Dikic *et al.*, 1996; Li *et al.*, 1996). RAFTK was shown to interact with several focal adhesion molecules such as paxillin, p130cas and HAF1 (Astier *et al.*, 1997a,b; Hiregowdara *et al.*, 1997; Salgia *et al.*, 1996). In this report, we have investigated the role of RAFTK in HRG-mediated signaling in breast cancer cells. HRG stimulates the tyrosine phosphorylation of RAFTK and induces the formation of a multiprotein

complex of RAFTK with p190 RhoGAP, RasGAP and ErbB-2. Characterization of the RAFTK association with p190 demonstrated a significant role for RAFTK in regulating the phosphorylation of p190 by Src and, therefore, mediating the association between p190 and RasGAP, thus linking both the Ras and Rho signaling pathways in breast cancer cells. Furthermore, we demonstrate the involvement of RAFTK in MCF-7 and MDA-MB-435 breast cancer cell invasion, suggesting that RAFTK can promote breast carcinoma migration.

Results

Tyrosine phosphorylation of RAFTK upon HRG stimulation leads to formation of a multiprotein complex

Since RAFTK is expressed in human breast epithelial cells and breast cancer tissues (data not shown), we investigated whether RAFTK is involved in the signaling pathways of the ErbB receptor family. We examined the effect of HRG stimulation on the tyrosine phosphorylation of RAFTK in the T47D human breast cancer cell line. Quiescent T47D cells were stimulated with 10 nM HRG for different time points (Figure 1a,b). As shown in Figure 1a, HRG induced the tyrosine phosphorylation of RAFTK within 2 min and the formation of a multiprotein complex. Maximal tyrosine phosphorylation of the proteins participating in this complex occurred between 2–6 h of HRG stimulation (Figure 1b). A high molecular weight tyrosine-phosphorylated protein (~190 kD) was co-immunoprecipitated with RAFTK and additional tyrosine-phosphorylated proteins of approximately 150–160 kD and 70 kD appeared to join this complex. When we compared the effect of HRG to that of EGF at different time points (0–120 min) and different concentrations (1.25–200 ng/ml), the RAFTK immunoprecipitates from EGF-stimulated T47D cell lysates showed no RAFTK

Figure 1 Tyrosine phosphorylation of RAFTK upon HRG stimulation of T47D cells. Quiescent T47D cells were stimulated with 10 nM HRG for different time points (a, b). Cell lysates were immunoprecipitated with anti-RAFTK antibodies or pre-immune serum (NRS) as a control, separated on 7% SDS-PAGE and blotted with 4G10 anti-phosphotyrosine antibodies (upper panels). The same blots were stripped and reprobed with anti-RAFTK antibodies (lower panels). The phosphorylated proteins p190 and p120 as well as RAFTK are indicated by arrows

We next examined whether the long-term phosphorylation is dependent on protein or RNA synthesis. T47D cells were stimulated with HRG for different time points (0–24 h) and treated either with cycloheximide or actinomycin-D which blocks protein and RNA synthesis, respectively. No significant effect on complex formation and phosphorylation of RAFTK and the participating proteins was observed (data not shown).

To identify the proteins that participate in the observed multiprotein complex that is formed upon HRG stimulation, several candidates were examined based on their molecular weight. Protein extracts from HRG-stimulated T47D cells were immunoprecipitated using anti-RAFTK antibodies, and blotted with the following specific antibodies for these focal adhesion molecules: talin, tensin, or vinculin. No association of these molecules with RAFTK was observed (data not shown). However, when the RAFTK immunoprecipitates were analysed by SDS-PAGE and blotted with anti-p190 RhoGAP (=p190) antibodies, the tyrosine-phosphorylated protein of p190 kD was identified as p190 RhoGAP (Figure 2a). Furthermore, the association of p190 and RAFTK was constitutive and independent of HRG stimulation. Immunoprecipitation with pre-immune serum (NRS) as a control showed no association with p190, indicating the specificity of this association (Figure 2a).

its participation in the RAFTK complex upon HRG stimulation (Figure 2b).

Several candidates for the 150–160 kD tyrosine-phosphorylated proteins were examined based on their molecular weights, including SOS 1 and p160Rock. We could not detect any association of these proteins with RAFTK. In addition, we examined whether the phosphorylated 160 kD protein is a cleavage product of p190. Our results indicated that the p160 kD is not a cleavage product of p190 (data not shown). Thus, the identity of the tyrosine-phosphorylated 160–170 kD protein remains unknown.

To further characterize the association of RAFTK with the p190 molecule, starved T47D cells were stimulated with HRG for different time points. Immunoprecipitates of p190 were analysed by SDS-PAGE, and reacted with anti-phosphotyrosine antibodies (4G10). The phosphorylation of p190 was observed within 5 min of HRG stimulation (Figure 3a). HRG induced the same pattern of complex formation of p190 with the 150–160 kD and 120–130 kD tyrosine-phosphorylated proteins as the RAFTK immunoprecipitates. Blotting of the p190 immunoprecipitates from the same experiment with anti-RAFTK antibodies confirmed the constitutive association of these two molecules (Figure 3b).

To further confirm the association of the p190 and RAFTK molecules, 293T cells were transiently co-transfected with different constructs of Flag-tagged RAFTK and HA-tagged p190. Reciprocal co-immunoprecipitations with anti-HA or anti-M2 (anti-Flag) antibodies, followed by immunoblotting with anti-M2 or anti-HA, respectively, were performed. Figure 4a demonstrated that the RAFTK wild-type (WT) molecule as well as the kinase mutant (KM) of RAFTK or the Src binding site-mutated RAFTK Y⁴⁰²→F (402) can bind equally to the wild-type p190 molecule as well as to the mutant p190-FF (point mutation in the RasGAP binding sites at tyrosine residues Y¹¹⁰⁵ and Y¹⁰⁸⁷→F (Hu and Settleman, 1997)).

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Figure 3 Association of RAFTK with p190 RhoGAP. (a) T47D cells were stimulated with 10 nM HRG for different time points. Cell lysates were immunoprecipitated with anti-p190 antibodies or with control antibody followed by immunoblotting with 4G10 (a). The same blot was stripped and reblotted with anti-p190 antibodies (b). The same immunoprecipitated samples were separated on SDS-PAGE and immunoblotted with anti-RAFTK antibodies (b)

Figure 4 Characterization of p190 RhoGAP association with RAFTK. 293T cells were co-transfected with the HA-tagged wild-type p190 molecule (190-WT) or with the double tyrosine (1087F, 1105F) mutant p190 (190-FF) and with the Flag-tagged wild-type RAFTK molecule (WT), kinase mutant (KM) or the Src binding site-mutated RAFTK molecule (402). Cell lysates were split into two portions: one half was immunoprecipitated with either anti-M2 antibodies or control antibody, followed by immunoblotting with anti-M2 as indicated (a), and the other half was reciprocally immunoprecipitated using either anti-M2 antibodies or control antibody followed by blotting with anti-HA antibodies (b). 293T cells were co-transfected with the Flag-tagged RAFTK molecules NKC and NKP (different truncated RAFTK molecules), and with a vector or with the HA-tagged double tyrosine mutant p190 (p190-FF). Cell lysates were precipitated using a GST-fusion protein of p120 RasGAP (SH2-SH3-SH2), with GST alone as a control or with p190-FF together with SH2-SH3-SH2, followed by blotting with M2 anti-Flag antibodies (c)

When cells were transfected with p190-WT alone as a control and immunoprecipitated with M2 antibodies, the p190 molecule was not detected using anti-HA antibodies (Figure 4b), indicating the specificity of the interactions observed. Reprobing of the same blots with the comparable HA or M2 antibodies showed equal amounts of transfected proteins (data not shown). Therefore, the association of RAFTK and p190 is not dependent on the association with either RasGAP (based on the results of the p190-FF mutant) or with c-Src (see mutant 402).

RasGAP association with RAFTK

To further analyse the association of RAFTK with RasGAP, we examined if the binding of RasGAP to RAFTK is indirect and mediated by p190. 293T cells were transiently co-transfected with RAFTK constructs in the presence or absence of the p190-FF (a mutant which cannot bind RasGAP). Cell lysates were incubated with a GST-fusion protein containing the SH2-SH3-SH2 domain of the p120 RasGAP

protein (Hu and Settleman, 1997) or with the GST-fusion protein alone as a control. The precipitated proteins were separated on SDS-PAGE and were immunoblotted using M2-tagged RAFTK antibodies. Expression of the p190-FF mutant did not affect the observed RAFTK-RasGAP association (Figure 4c), although p190-FF was shown to be capable of binding to RAFTK (Figure 4a). These results suggest that the association of RasGAP and RAFTK does not depend on the binding of RasGAP to p190.

RAFTK association with ErbB-2

Since RAFTK is tyrosine-phosphorylated upon HRG stimulation, we sought to characterize the possible interaction of RAFTK with the ErbB-2 receptor. As shown in Figure 5a, ErbB-2 was co-immunoprecipitated with RAFTK shortly after HRG stimulation (5 min). Maximal association was observed after 30 min, and decreased after 2 h. To analyse whether this interaction is direct or mediated by Src, 293T cells were transiently co-transfected with the transformed ErbB-2/neu (neu*) cDNA expression construct that encodes for the constitutively phosphorylated ErbB-2 receptor (point mutation Val #664→Glu) (Ben-Levy et al., 1992, 1994), and with the Flag-tagged RAFTK wild-type molecule (WT), or with the Src binding site-mutated RAFTK Y⁴⁰²→F (402). Figure 5b demonstrates that mutant RAFTK 402 did not bind ErbB-2, suggesting that the binding of RAFTK to ErbB-2 is indirect and is mediated by Src.

RAFTK mediates the tyrosine phosphorylation of p190 by Src

To determine the possible role of RAFTK in this multiprotein complex, we examined the effect of RAFTK on the tyrosine phosphorylation of p190. 293T cells were transiently co-transfected with HA-tagged p190 and Flag-tagged wild-type RAFTK (WT), kinase mutant (KM) or the Src binding mutant (402) with different combinations of the activated neu receptor (neu*) and v-Src, as indicated. Total cell lysates were prepared and immunoprecipitated with anti-HA antibodies (Figure 6a,b,d) and with anti-M2 antibodies (Figure 6c). Tyrosine phosphorylation of the p190 molecule was examined by blotting the anti-HA immunoprecipitates with anti-phosphotyrosine antibodies (4G10). Figure 6a shows that p190 can be phosphorylated in the presence of RAFTK-WT, and its phosphorylation is not affected by the presence of the kinase-dead RAFTK (lanes 2 and 3, respectively). However, in the presence of the RAFTK-402 molecule (mutated Src binding site) (lane 4), the tyrosine phosphorylation of p190 was significantly reduced (compare lane 4 with lanes 2 and 3). This effect was even clearer in the presence of the activated neu receptor (lanes 6 and 7 compared to lane 8). These results suggest that RAFTK kinase activity is not necessary for p190 phosphorylation. However, Src binding to RAFTK is essential for the phosphorylation of p190.

Overexpression of v-Src leads to the tyrosine phosphorylation of p190 (Figure 6a, lane 9). The presence of RAFTK-WT with v-Src augmented p190

Figure 5 RAFTK association with ErbB-2. (a) T47D cells were stimulated with 10 nM HRG for different time points. Cell lysates were immunoprecipitated with anti-RAFTK antibodies or with pre-immune serum (NRS), and separated on 7% SDS-PAGE. The blot was then incubated with anti-ErbB-2 antibodies. (b) 293T cells were co-transfected with the activated neu receptor (neu*) and with two different Flag-tagged RAFTK constructs: Src binding site mutant (402) or with wild-type (WT). Cell lysates were immunoprecipitated with anti-ErbB-2 antibodies (anti-neu, Ab-4) and then blotted with M2 antibodies (a), or reciprocally were immunoprecipitated with M2 antibodies, followed by immunoblotting with anti-ErbB-2 antibodies (b)

Figure 6 RAFTK mediates the phosphorylation of p190 by Src. 293T cells were co-transfected with the HA-tagged p190 construct and with different combinations of Flag-tagged wild-type RAFTK molecule (WT), kinase mutant (KM), Src binding site-mutated RAFTK (402), activated neu receptor (neu*) and v-Src as indicated above. Cell lysates were split into two portions and immunoprecipitated with either anti-HA antibodies (a, b and d) or with anti-M2 antibodies (c). Immunoblotting of the anti-HA precipitates with 4G10 antibodies shows tyrosine phosphorylation of the p190 molecule (a). The same blot was stripped and reblotted with anti-HA antibodies to demonstrate the protein levels of p190 (b). The same blot was stripped again and reblotted with anti-RasGAP antibodies (d). Immunoprecipitation with anti-M2 antibodies followed by Western blotting with anti-M2 antibodies indicates the expression levels of the RAFTK constructs (c)

phosphorylation (lane 10), while the 402 mutant of RAFTK reduced this effect significantly (lane 11). The effect of RAFTK on the tyrosine phosphorylation of p190 by v-Src was enhanced in the presence of the activated neu* receptor (compare lanes 10 and 11 to lanes 13 and 14). The same effect was also observed when the c-Src construct was used in these transfection studies (data not shown). The control untransfected cells had no background (lane 15). Reblotting of the same blots with anti-HA antibodies demonstrated comparable amounts of the p190 molecule (Figure 6b), and immunoprecipitation of the same samples with M2 antibodies (which detect transfected exogenous RAFTK protein), followed by immunoblotting with M2, confirmed the expression of equal levels of RAFTK constructs in the specific samples (Figure 6c).

These results demonstrate that the RAFTK molecule serves as a mediator that binds p190 constitutively, and that upon activation with HRG, RAFTK binds to Src in tyrosine residue 402. This binding brings Src into proximity with its substrate, p190. Mutation of this site (402) significantly reduced the phosphorylation of p190 by Src. No effect on the phosphorylation of p190 was observed in the presence of the kinase-dead RAFTK mutant (lanes 3 and 7). Therefore, we conclude that RAFTK does not phosphorylate p190 directly, but is essential for the phosphorylation of p190 by Src.

We next examined the effect of RAFTK presence on binding to RasGAP. The same blots were stripped and reblotted with anti-RasGAP-specific antibodies. As expected, correlation between the tyrosine phosphorylation of p190, which is enhanced by the presence of

RAFTK, and the association of p190 with RasGAP was observed (Figure 6d).

RAFTK mediates activation of MAP kinase by HRG

Since we have shown that RAFTK is tyrosine-phosphorylated upon HRG stimulation, we examined the possibility that RAFTK can act downstream of ErbB-2 in the cascade of events leading to MAP kinase activation. 293T cells were transiently co-transfected with the HA-tagged Erk construct and with different combinations of RAFTK wild-type (WT) or kinase mutant (KM), v-Src, activated neu receptor (neu*), and constitutively activated *ras* (V12) or dominant negative *ras* (N17), as indicated. Cell lysates were immunoprecipitated with anti-HA antibodies and subjected to *in vitro* kinase assay using MBP as Erk substrate (Figure 7). The results demonstrate that RAFTK-WT but not RAFTK KM can activate Erk (compare lane 2 to lane 3, respectively). Strong activation of Erk is observed by the activated neu receptor (lane 4). Blocking of neu activation by the kinase mutant of RAFTK (lane 6) indicated that RAFTK is downstream of neu in Erk activation. The presence of dominant negative *ras* N17 completely blocked activation by neu and RAFTK-WT (lane 8), indicating that *ras* is downstream of neu and that RAFTK molecules are present in this pathway. By using the Src kinase mutant (SKD), we demonstrate

that Src had no effects on the activation of Erk by neu* or neu and RAFTK (lanes 9 and 10, respectively). Moreover, activation of Erk by v-Src was unaffected by *ras* N17 (lane 7). Activation of Erk by *ras* V12 was not affected by the Src mutant (lane 11). Therefore, RAFTK mediates the activation of Erk by neu, and this pathway involves *ras* molecules but not Src.

RAFTK involvement in breast cancer cell invasion

The interaction of RAFTK with p190 RhoGAP suggests that RAFTK may play a role in regulating the function of Rho family members and, in doing so, could be involved in modifying the actin cytoskeleton to promote breast carcinoma motility and invasion. To examine this possibility, we investigated the contribution of RAFTK to *in vitro* MDA-MB-435 breast carcinoma cell invasion. These cells were chosen based on the fact that they do not endogenously express the RAFTK protein and do require Rho function for invasion (data not shown). MDA-MB-435 cells were transiently transfected with the RAFTK-WT cDNA, as well as with the mutant RAFTK cDNAs that were described previously. The ability of MDA-MB-435 cells expressing RAFTK proteins to invade through a Matrigel-coated filter was examined. As shown in Figure 8a, expression of RAFTK-WT increased MDA-MB-435 cell invasion by approximately twofold. In contrast, expression of the kinase-inactive RAFTK (KM) or the Src binding site mutant RAFTK (402) did not increase this cell invasion.

In order to elucidate the role of RAFTK in HRG-induced cell migration, MCF-7 cells were transiently transfected with various RAFTK cDNA constructs as indicated in Figure 8b. After 48 h, HRG (10 nM) was added for 16 h and cell migration was analysed using Boyden Chambers. As shown in Figure 8b, expression of RAFTK-WT enhanced the migration of MCF-7 cells upon HRG stimulation, and the expression of the constitutively-active (CA) RAFTK molecule increased the migration of MCF-7 cells by threefold, even in the absence of HRG stimulation. Expression of the Src binding site mutant (402) or the kinase-inactive RAFTK (KM) inhibited the HRG-stimulated migration of MCF-7 cells. These results demonstrate that RAFTK can promote breast carcinoma cell invasion and that this function requires the kinase activity of RAFTK as well as its ability to bind and activate Src.

Figure 7 RAFTK mediates activation of MAP kinase by neu*. 293T cells were co-transfected with HA-tagged Erk construct and with different combinations (as indicated above) of: FLAG-tagged RAFTK wild-type (WT) or kinase mutant (KM) and activated neu receptor (neu*), v-Src or a dominant negative mutant of Src (SKD), constitutively activated *ras* (V12) or a dominant negative *ras* construct (N17). Cell lysates were immunoprecipitated using anti-HA antibodies. Then the precipitates were subjected to *in vitro* kinase assay using MBP as substrate. Samples were separated on SDS-PAGE and exposed to autoradiography (a). Lane 1: control without the substrate MBP. One-third of the same immunoprecipitates (before kinase assay samples) were separated on 8% SDS-PAGE and blotted with anti-HA antibodies to show the expression levels of the HA-tagged Erk construct (b)

Discussion

In this study, we have investigated the role of RAFTK in HRG-mediated signaling in breast cancer cells. HRG stimulated the tyrosine phosphorylation of RAFTK and the formation of a multiprotein complex in T47D cells. The proteins participating in this complex include p190 RhoGAP, p120 RasGAP and ErbB-2. Characterization of the RAFTK and p190 interaction revealed that RAFTK plays an essential role in mediating the tyrosine phosphorylation of p190 by Src, and thus in controlling the association of p190 and RasGAP.

We observed a rapid tyrosine phosphorylation of RAFTK as well as p190 upon HRG stimulation of T47D cells. This phosphorylation was maximal between 2–6 h of HRG treatment and lasted for 6 h.

Figure 8 RAFTK involvement in MDA-MB-435 cell invasion by transient transfection. (A) Analysis of RAFTK involvement in MDA-MB-435 cell invasion by transient transfection. MDA-MB-435 cells were assayed for their ability to invade Matrigel after transient transfection with RAFTK cDNAs. Cells were transiently transfected with 1 μ g pCS2-(n) β -Gal and 4 μ g of either the vector alone, wild-type RAFTK (WT), RAFTK-R402 (402) or RAFTK-R457 (KM). Transfected cells (10^6) were added to the upper wells of the Matrigel-coated Transwell chambers and conditioned NIH3T3 medium was added to the bottom wells. After 6 h at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in Materials and methods. The data shown are the mean values ($\pm$ s.e.m.) of four experiments done in triplicate (a). Cell lysates (10^6) from transfected cells were immunoprecipitated with M2 antibody and separated on 7% SDS-PAGE. The blot was then incubated with anti-RAFTK antibodies (b). (B) Effect of RAFTK on the migration of MCF-7 cells. Cells were transiently transfected with RAFTK cDNA constructs, as indicated, for 48 h. HRG (10 nM) was either added or not to the cultures for 16 h and cell migration was analysed using Boyden Chambers. CA=constitutively active RAFTK

The unusual prolonged phosphorylation of RAFTK (by 2 h and up to 6 h), and the protein-complex associated with the phosphorylated RAFTK, were not dependent on protein or RNA synthesis, as indicated by the cycloheximide and actinomycin-D experiments.

Concomitantly with the enhanced phosphorylation of RAFTK observed between 2–6 h of stimulation, we demonstrated that HRG also induced the formation of a multiprotein complex. Based on our results, p190 RhoGAP is associated with RAFTK. FAK was reported to be associated with an approximately 200 kD protein in NIH3T3 cells upon PDGF stimulation. Although the identity of this protein is unknown, the authors excluded the possibility that this protein might be the p190 RhoGAP molecule (Chen and Guan, 1996). Further characterization of the RAFTK-p190 association revealed that RAFTK was constitutively associated with p190, and that this association was not mediated by either Src or RasGAP (Figure 4).

Analysis of the tyrosine phosphorylation pattern of p190 upon HRG stimulation revealed similar kinetics to RAFTK (prolonged phosphorylation) (Figure 3). A similar prolonged tyrosine phosphorylation of p190 was reported when human leukemia cells were exposed to a variety of differentiation agents such as PMA, DMSO and retinoic acid (Cheng *et al.*, 1995). Thus, the demonstrated role for Rho and Rac in the formation of actin filament structures suggests that the observed changes in p190 phosphorylation may be integrally involved in the reorganization of the cytoskeleton during cell cycle progression and differentiation (Cheng *et al.*, 1995).

The association of RasGAP with RAFTK upon HRG stimulation was observed in our study. This association was not dependent on the p190-RAFTK association since the RasGAP-RAFTK complex was formed in the presence as well as the absence of the mutated p190 molecule (FF), which is not able to bind RasGAP (Figure 4). Interestingly, association of FAK (focal adhesion kinase) which is closely related to RAFTK, with RasGAP, was reported. During brain development, FAK was shown to interact with both SH2 domains of RasGAP (Serpente *et al.*, 1996). Recently Graf, an SH3 domain-containing GAP molecule for Rho and CDC42, was isolated and shown to associate with FAK as well as with RAFTK/CAK- β via its SH3 domain (Hildebrand *et al.*, 1996; Ohba *et al.*, 1998; Taylor *et al.*, 1998). Thus, our data indicate that the RAFTK-mediated association between GAP molecules and focal adhesion molecules may be a site of convergence for growth factor signaling and Rho-mediated cytoskeletal changes.

This study demonstrates that the tyrosine phosphorylation of p190 by Src is mediated by RAFTK. It was previously reported that p190 is a preferred substrate for Src. Here, we show that p190 is constitutively bound to RAFTK, and that binding of RAFTK to Src enhances the phosphorylation of p190. Abolishment of the binding of Src to RAFTK, using Src binding site-mutated RAFTK (402), caused a significant attenuation of the tyrosine phosphorylation of p190. This effect was more significant when cells were transfected with the activated neu receptor. These observations suggest that upon HRG stimulation as well as upon activation of the neu receptor, Src is recruited to RAFTK, into proximity with its substrate p190 (Figure 6). Furthermore, we show that, by enhancing the tyrosine phosphorylation of p190, RAFTK can mediate the binding of p190 to RasGAP (Figure 6d). It has been demonstrated previously that p190 is a major substrate for tyrosine phosphorylation in cells that are oncogenically transformed by tyrosine

kinases. Interestingly, p190-B gene expression is regulated during mammary development and p190-B is upregulated in a subset of experimentally-induced rodent tumors (personal communication, Geetika Chakravarty and Jeff Rosen, Baylor University School of Medicine). This suggests that formation of the RasGAP-p190 complex may be a critical step in the transformation process (Ellis *et al.*, 1990).

HRG stimulation of breast cancer cells enhances activation of PI3-kinase and MAP kinase signaling pathways. MAP kinase plays a crucial role in HRG signaling during growth and differentiation of mammary epithelial cells. Despite the widely acknowledged role of the ErbB/HRG pathway in breast cancer progression, the signaling pathway(s) involved remains elusive. Here we show that RAFTK is involved in the activation of the ErbB-2 receptor by HRG, which leads to MAP kinase activation. The involvement of RAFTK is not mediated by Src, but is mediated by the *ras* molecules. Thus, the involvement of RAFTK in HRG stimulation of MAP kinase is upstream of *ras*.

Our data support a role for RAFTK in breast carcinoma cell invasion. These results are novel as they are the first to demonstrate that RAFTK can function in promoting cell motility. The involvement of RAFTK in cell migration is not surprising given the numerous studies that have demonstrated a role for the RAFTK homolog, p125-FAK, in cell migration. The ability of RAFTK to promote motility may be cell-type dependent because exogenous expression of RAFTK was not sufficient to fully rescue the migration of FAK null fibroblasts (Rozengurt and Rodriguez-Fernandez, 1997). We observed that RAFTK-dependent invasion requires both the kinase activity of the protein as well as its ability to associate with and activate Src. RAFTK recruitment and activation of Src are also required for p190 RhoGAP phosphorylation and association with RasGAP, which supports the potential involvement of this complex in regulating invasion. Further studies will be required to elucidate the specific contribution of RAFTK to carcinoma cell motility and invasion.

In summary, we show that upon HRG activation, RAFTK plays an essential role in the complex formation with ErbB-2, and in modulating the association of RasGAP-p190 RhoGAP, thus providing a link between ErbB-2 activation and focal adhesion/cytoskeletal signaling in breast cancer cells (McWhirter and Wang, 1991). In addition, RAFTK can play an essential role in modulating the RasGAP-p190 complex by mediating the phosphorylation of p190 by Src. It was previously reported that the two tyrosine residues Y¹⁰⁸⁷ and Y¹¹⁰⁵ within the p190 molecule serve as binding sites for RasGAP, and that these residues also serve as *in vivo* substrates for Src and for c-Abl (Hu and Settleman, 1997). Therefore, we suggest that RAFTK serves as a modulator molecule that is constitutively bound to p190. Upon HRG stimulation, RAFTK recruits Src, thus bringing it into close proximity with p190. Thus, RAFTK mediates the phosphorylation of p190 and, therefore, can control its association with RasGAP.

The small GTPases, Rho, Rac, CDC42 and Ras cooperate to promote cell movement and regulate cell migration (Nobes and Hall, 1999). Using an *in vitro* wound healing assay, CDC42 and Rac were found to be essential for cell polarity and for producing the leading edge protrusions required for forward movement. Rho

was found to be required to maintain cell adhesion during movement, and Ras regulates focal adhesion and stress fiber turnover which is essential for cell movement. In this study, we demonstrate the involvement of RAFTK in regulating breast cancer cell invasion. In addition, we demonstrate that RAFTK mediates activation of MAP kinase by HRG as well as the interaction of RhoGAP-RasGAP. Thus, these results suggest that RAFTK serves as an integration site between GAP molecules and HRG-mediated signaling and thereby can mediate the cytoskeletal changes initiated by HRG through the ErbB-2 receptor in breast cancer cells.

Materials and methods

Plasmid construction and mutagenesis

RAFTK cDNA constructs were described previously (Li *et al.*, 1996; Hiregowdara *et al.*, 1997). Construct coding for the activated neu receptor (neu*) was kindly provided by Dr Yosef Yarden (Ben-Levy *et al.*, 1992, 1994). The HA-tagged constructs of p190 in the RCMV vector, HA-190-WT as well as the RasGAP binding site mutant HA-190-FF (Y¹⁰⁸⁷→F and Y¹¹⁰⁵→F) were described previously (Hu and Settleman, 1997).

Cell culture and transfection

The T47D and MCF-7 human breast cancer cell lines and the 293T human embryonic kidney cells were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). For stimulation studies, 5 × 10⁶ cells/plate were seeded and grown overnight. Prior to stimulation with HRG (rHRGβ1 177-244, kindly provided by Mark X Sliwkowski, Genentech Inc., San Francisco, CA, USA) or with EGF (Collaborative Biomedical Products, Bedford, MA, USA), cells were starved overnight in media containing 1% fetal bovine serum and then for 4 h in serum-free medium. For treatment with cycloheximide (10 μg/ml), cells were incubated for 1 h before stimulation with cycloheximide (Sigma, St. Louis, MO, USA), and then HRG was added. Actinomycin-D (Sigma) (5 μg/ml) and HRG were added to the cells for the indicated time points. Transfection of T47D and MCF-7 cells was performed using Lipofectamine plus reagent (Life Technologies, Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. For 293T cells, the calcium phosphate method was used. Cells were harvested 48 h post transfection.

Immunoprecipitation and immunoblotting

For protein analysis, cells were harvested and then lysed in ice-cold modified RIPA buffer. For immunoprecipitation, lysates were incubated for 1 h at 4°C with: polyclonal anti-RAFTK antibodies (4250), pre-immune rabbit serum, monoclonal anti-p190 RhoGAP (Transduction Laboratories, Lexington, KY, USA), monoclonal anti-RasGAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-neu (Ab-4) (Oncogene Sciences), monoclonal anti-HA probe (Santa Cruz Biotechnology) or with monoclonal anti-M2 (Kodak). Next, protein G-Sepharose (Pierce, Rockford, IL, USA) was added to the samples for 90 min. The samples were then washed three times with RIPA lysis buffer (described above), and resuspended in 1 × SDS sample buffer. Samples were resolved by 7% SDS-PAGE, and the gel was transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% non-fat milk in TBS-T for 1 h and then incubated for 1 h or overnight with primary antibodies such as: anti-phosphotyrosine (4G10)–kindly provided by Dr Brian J Druker (Division of

Hematology and Medical Oncology, Oregon Health Sciences University, Portland). The blots were washed four times for 15 min each, then secondary antibody was added for 1 h and blots were developed using the ECL system (Amersham Pharmacia).

MAP kinase assay

Cell lysates from HA-tagged Erk-transfected cells were immunoprecipitated using anti-HA antibodies. Then kinase buffer was added (200 mM HEPES, pH 7.2, 100 mM MgCl₂, 1 mg/ml BSA, 30 mM BME) and the samples were subjected to kinase assay for 10 min at 30°C using MBP (Sigma) as substrate. Reaction was terminated by adding sample buffer, then boiling and separating the samples on 12% SDS-PAGE.

Invasion assays

The MDA-MB-435 breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository at Georgetown University. Cells were co-transfected with 1 µg pCS2-(n)β-Gal and the cDNAs specified in the figure legends using Lipofectamine (Gibco) according to the manufacturer's instructions. Twenty-four hours after transfection, Matrigel invasion assays were performed as described previously (Shaw et al., 1997) using 6.5 mm Transwell chambers (8 µm pore size; Costar). Matrigel was diluted in cold distilled water, added to the upper wells of the Transwell chambers (2 µg/well), and dried in a sterile hood. The Matrigel was reconstituted with medium for 1 h at 37°C before the addition of cells. Cells were resuspended in serum-free medium containing 0.1% BSA at a concentration of 1–

2 × 10⁶ cells/ml, and 100 µl of the cell suspension was added to each well. Conditioned NIH3T3 medium (600 µl) was added to the bottom wells of the chambers. After 6 h, the cells that had not invaded were removed from the upper face of the filters using cotton swabs. The cells that had invaded to the lower surface of the filters were fixed, stained, and counted. All of the β-galactosidase (β-Gal)-positive cells were counted for each well.

Migration of MCF-7 cells was performed as described (Adam et al., 1994). Results are expressed as the percentage of migrating cells as compared with the total number of cells (cells present in all Z-sections) and represent the means ± s.e.

Abbreviations

aa, amino acids; FAK, focal adhesion kinase; GAP, GTPase-activating protein; GST, glutathione S-transferase; HRG, heregulin; IP, immunoprecipitation; RAFTK, related adhesion focal tyrosine kinase; WB, Western blot.

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