Heregulin and HER2 signaling selectively activates c-Src phosphorylation at tyrosine 215

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Abstract To elucidate the molecular mechanisms by which human epidermal growth factor receptor/heregulin (HER2/HRG) influence the migratory potential of breast cancer cells, we have used phospho-specific antibodies against c-Src kinase and focal adhesion kinase (FAK). This study establishes that HER2/HRG signaling selectively upregulates Tyr phosphorylation of c-Src at Tyr-215 located within the SH2 domain, increases c-Src kinase activity and selectively upregulates Tyr phosphorylation of FAK at Tyr-861. HER2-overexpressing tumors showed increased levels of c-Src phosphorylation at Tyr-215. These findings suggest that HER2/HRG influence metastasis of breast cancer cells through a novel signaling pathway involving phosphorylation of FAK tyrosine 861 via activation of c-Src tyrosine 215.

Key words: Receptor tyrosine kinase; Cytoplasmic tyrosine kinase; Growth factor signaling

1. Introduction

Human epidermal growth factor receptor 2 (HER2/neu) [1], heregulin (HRG), a combinatorial HER ligand [2], and Src kinases [3,4] have been implicated in the regulation of breast cancer progression. Growth factor signaling regulates c-Src kinase activity by altering the tyrosine phosphorylation of specific Tyr residues. Tyrosine phosphorylation at residues Tyr-215 and Tyr-418 increases c-Src kinase activity, while Tyr phosphorylation at Tyr-529 down regulates c-Src kinase activity [4]. Focal adhesion kinase (FAK), a non-receptor tyrosine kinase and substrate of c-Src present in focal adhesions, is implicated in the regulation of cell motility, adhesion, and anti-apoptotic signaling [5,6]. Growth factors induce phosphorylation of FAK on a number of Tyr residues and each of the FAK Tyr residues has been implicated in generating a distinct signal. For example, FAK Tyr-397 in recruiting c-Src, phosphoinositide 3′-kinase and p130CAS to focal adhesions; FAK Tyr-576 and FAK Tyr-577 in upregulating FAK kinase activity [7]; FAK Tyr-925 in activating the Ras-mitogen-activated protein kinase pathway [8]; and FAK Tyr-861 in Ras-mediated transformation [9]. Cells lacking FAK are refractory to platelet-derived growth factor- and epidermal growth factor-mediated motility signals [10].

Growth factor-mediated formation of motile structures involves regulation of FAK tyrosine phosphorylation [11]. Interestingly, HRG or activated HER2 reorganize the cytoskeleton, and increase the metastatic potential of breast cancer cells without an increase in the total tyrosine phosphorylation of FAK, suggesting HER2 might use a distinct pathway to regulate FAK [12,13]. However, very little information is known on the molecular mechanisms by which HER2 and HRG regulate Src and FAK signaling to alter the metastatic potential of breast tumor cells. The results from this study suggest that HRG and HER2 signaling selectively upregulates tyrosine phosphorylation of c-Src at Tyr-215 located within the SH2 domain, increases c-Src kinase activity and selectively upregulates tyrosine phosphorylation of FAK at Tyr-861.

2. Materials and methods

2.1. Cell cultures, reagents and plasmids

MCF-7 human breast cancer cells, NIH 3T3 cells, and B104 (NIH 3T3 cells expressing kinase-activated HER2) [13] were maintained in Dulbecco's modified Eagle's medium–F12 medium (1:1) supplemented with 10% fetal calf serum. Phospho-specific antibodies against FAK and Src were purchased from Biosource (Camarillo, CA, USA). Antibodies against HER2 (#MS325-P) and recombinant HRG β-1 were purchased from Neomarkers (Fremont, CA, USA). Antibodies against FAK (#F2918) and vinculin (#V913) were purchased from Sigma (St. Louis, MO, USA). c-Src WT cDNA expression vector (#21-114) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Src mutant Y215F was constructed using the site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) using the following primers: forward, AGCGGCGGTTTCTTCATCACCTCCCGCACCCAG and reverse, CTGGGTGCGGGAGGTGATGAAGAAACCGCCGCT.

2.2. Cell extracts, immunoblotting and immunoprecipitation

MCF-7 cells were serum-starved for 48 h and treated with different concentrations of HRG (0.01 nM, 0.1 nM, or 1.0 nM). To prepare cell extracts, cells were washed three times with phosphate-buffered saline (PBS) and then lysed with RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 1×protease inhibitor cocktail (Roche Biochemical) and 1 mM sodium vanadate) for 15 min on ice.

2.3. Src kinase assays

In vitro Src kinase assays were performed following the previously described protocol [14]. Briefly, c-Src kinase was immunoprecipitated from 400 μg of cell lysates treated with or without HRG. Immunoprecipitates were washed three times with Triton X-100 lysis buffer and three times with kinase buffer (20 mM HEPES pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol) and kinase reactions were performed using 10 μg of acid-denatured enolase as a substrate at 30°C for 30

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Abbreviations: FAK, focal adhesion kinase; HER, human epidermal growth factor receptor; HRG, heregulin

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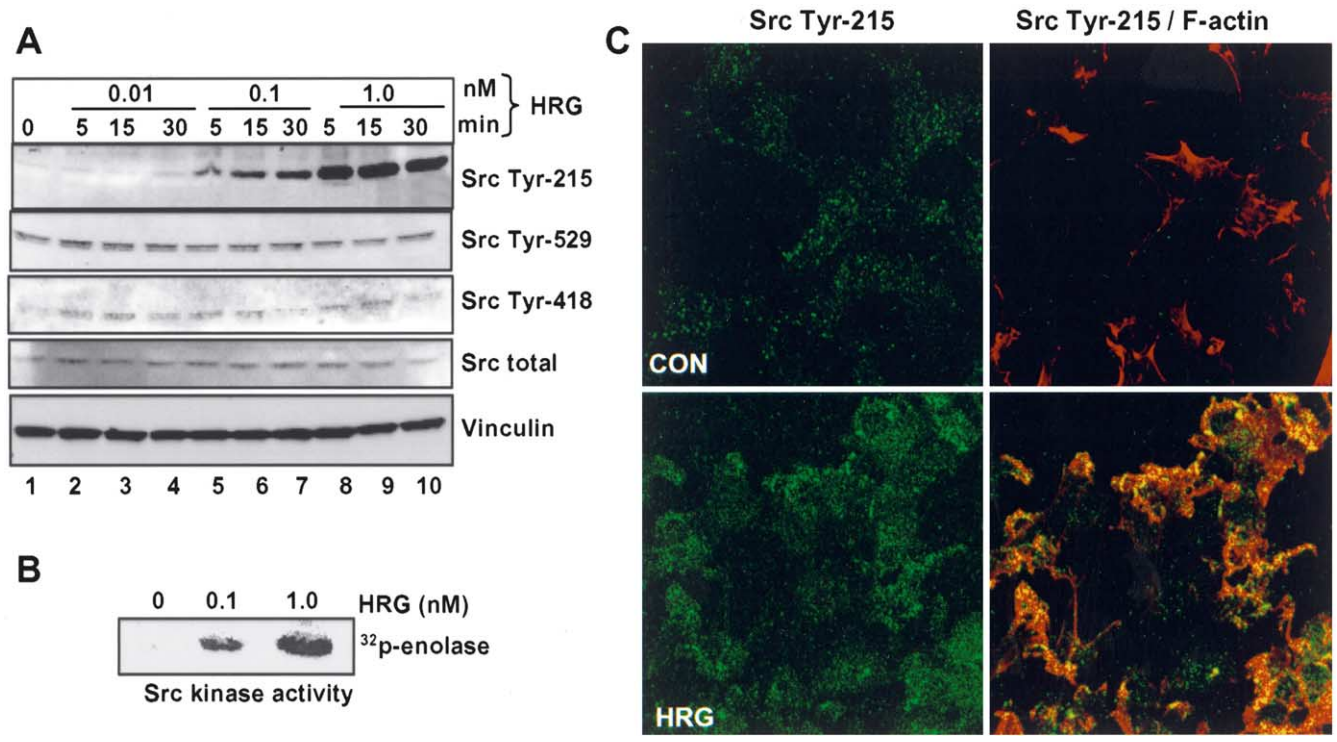

Fig. 1. HRG-induced Tyr phosphorylation of Src. A: Immunoblot analysis. MCF-7 cells were treated with 0.01 nM, 0.1 nM, or 1 nM HRG for the indicated times, and total lysates (200 μg) were analyzed by immunoblotting with site-specific antibodies that recognize distinct Tyr sites on Src kinase. B :In vitro kinase analysis. MCF-7 cells were treated with or without HRG and kinase assays were performed using enolase as a substrate. C: Confocal microscopy analysis. Localization of Src Tyr-215 was studied in MCF-7 cells treated with or with out HRG for 30 min. Src Tyr-215 is shown in green and phalloidin, a marker of F-actin-containing structures, is shown in red. Con indicates serum-starved MCF-7 cells not treated with HRG.

min, and visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by autoradiography.

2.4. Immunofluorescence studies and confocal microscopy

For indirect immunofluorescence studies, cells were blocked by incubation with 10% normal goat serum in PBS for 1 h at ambient temperature. Cells were then incubated for 1 h at ambient temperature with polyclonal antibodies against FAK Tyr-861 or Src Tyr-215 and with monoclonal antibodies against vinculin. After four washes with PBS, cells were incubated with ALEXA-488- or fluorescein isothiocyanate-conjugated goat anti-mouse IgG or with ALEXA-546-conjugated goat anti-rabbit IgG (1:100 dilution, Molecular Probes, Eugene, OR, USA) in 10% normal goat serum (in PBS). Control cells were treated only with the secondary antibody. Slides were analyzed by confocal microscopy.

3. Results

3.1. HER2/HRG signaling uniquely upregulates Src phosphorylation at Tyr-215

To examine the possible participation of Src kinase in HER2/HRG signaling, we utilized three well-characterized phospho-specific antibodies [15–17], which uniquely recognize Tyr-215, Tyr-418 and Tyr-529 on Src. We used MCF-7 breast cancer cells, which are known to express HER2 and HER3. MCF-7 breast cancer cells were treated with HRG, a ligand that is known to activate the HER2 pathway [18,19]. HRG selectively enhanced c-Src Tyr-215 phosphorylation in a dose- and time-dependent manner (Fig. 1A). The highest level of phosphorylation was observed at a concentration of 1 nM, which was previously shown to elicit a migratory phenotype in breast cancer cells [12]. Interestingly, there was no significant change in the phosphorylation of c-Src at Tyr-418, which was earlier linked to activation of Src kinase by a number of physiological signals [4]. Increased c-Src Tyr-215 phosphorylation correlated well with Src kinase activity as assessed by in vitro kinase assay (Fig. 1B). Confocal microscopy analysis of HRG-treated MCF-7 cells also demonstrated elevated c-Src Tyr-215 phosphorylation (Fig. 1C, bottom panel, green color). HRG treatment significantly induced the formation of motile structures such as ruffles, and importantly, a significant pool of activated c-Src Tyr-215 was localized in these structures. These results suggest that HRG may activate c-Src kinase via phosphorylation of Tyr-215 and that functionally activated c-Src kinase localizes to ruffles.

3.2. HRG induces phosphorylation of FAK at Tyr-861 in a c-Src kinase-sensitive manner

We next examined the functional consequence of HRG-induced stimulation of c-Src kinase by examining the phosphorylation status of a known downstream c-Src substrate, FAK. We used several commercially available Tyr site-specific antibodies against FAK Tyr sites [9]. We did not observe any significant increase in the phosphorylation of FAK at autophosphorylation site Tyr-397 (Fig. 2A). We also did not notice any increase in the Tyr phosphorylation of FAK Tyr-577, Tyr-925, rather, we observed a slight decrease in the level of tyrosine phosphorylation of these sites with an increase in HRG dose. Interestingly, HRG transiently stimulated the level of Tyr phosphorylation of FAK Tyr-861 in a dose-depen-

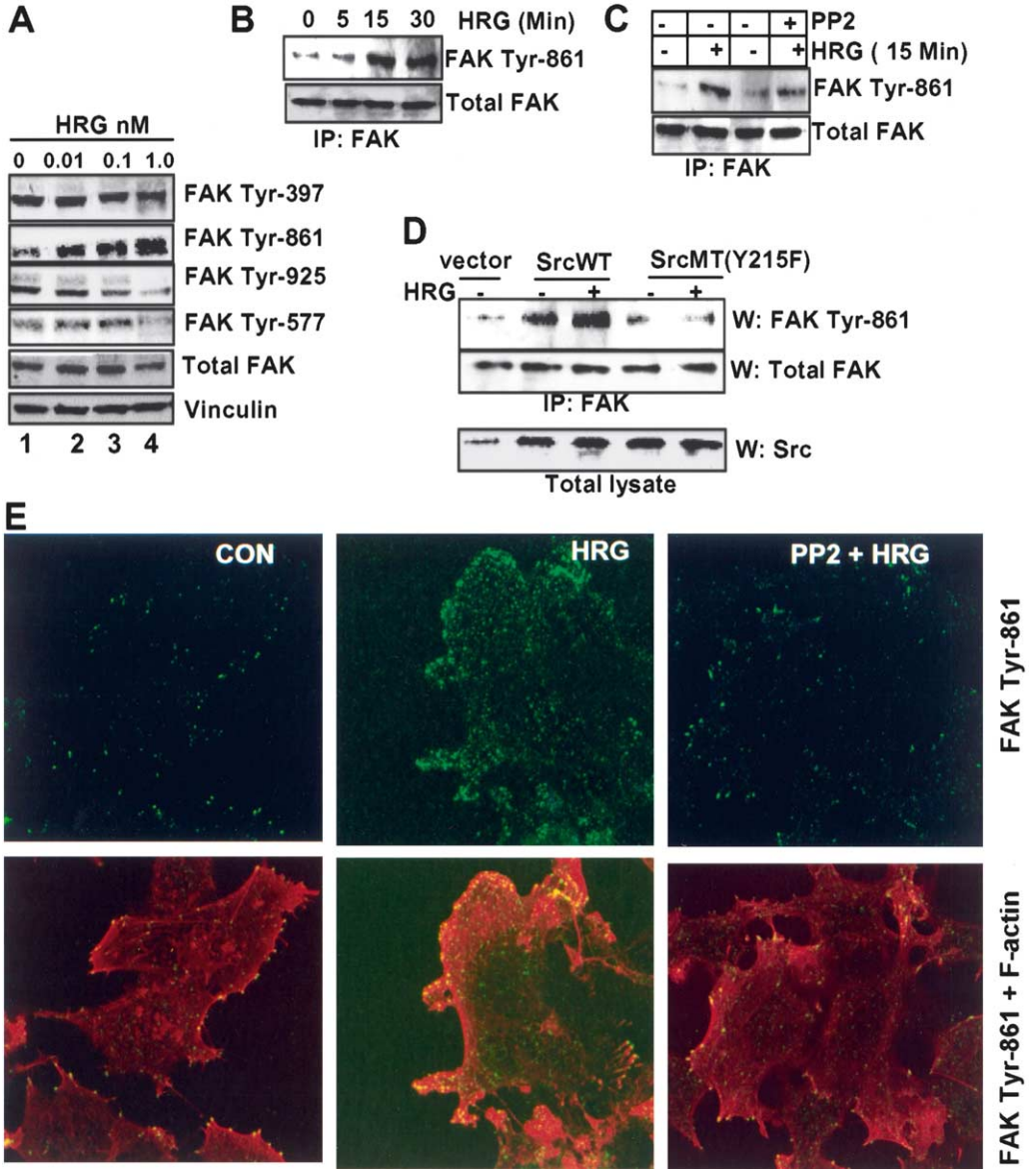

Fig. 2. HRG-induced tyrosine phosphorylation of FAK. A: Western blot analysis. MCF-7 cells were treated with 0.01 nM, 0.1 nM or 1 nM HRG for 30 min, and total lysates (200 μg) were analyzed by Western blotting with site-specific antibodies that recognize distinct sites on FAK. B: MCF-7 cells were treated with 1 nM HRG for indicated periods of time, equal amount of protein was immunoprecipitated with FAK antibody. Blots were probed with phospho-specific antibody against FAK Tyr-861. Blot was stripped and reprobed with FAK antibody C: MCF-7 cells were pretreated with or without the Src inhibitor PP2 for 30 min and then stimulated with 1 nM HRG for 15 min. Cell lysates were immunoprecipitated with FAK antibody, Western-blotted with phospho-specific FAK Tyr-861 antibody. D: MCF-7 cells were transfected with vector, Src wild type or Src mutant (SrcY215F) using Fugene-6 reagent in serum-free medium. After 24 h, cells were treated with 1 nM HRG for 15 min and equal amount of cell lysate was immunoprecipitated with FAK antibody followed by Western blotting with a phospho-specific antibody against FAK Tyr-861. Total lysate was run on a separate gel and analyzed by Western blotting using Src antibody (bottom panel) to visualize the expression of transfected Src constructs (lanes 2–5) compared to vector-transfected control (lane 1). E: Confocal microscopy analysis. Localization of FAK Tyr-861 was studied in MCF-7 cells treated with or without HRG for 30 min. As indicated some cells were pretreated with the Src inhibitor PP2 for 30 min before the addition of HRG. FAK Tyr-861 is shown in green, and phalloidin, a marker of F-actin-containing structures, is shown in red. Con indicates serum-starved MCF-7 cells not treated with HRG.

dent manner (Fig. 2A). HRG increased FAK Tyr-861 phosphorylation in a time-dependent manner with maximal activation at 15 min (Fig. 2B). The HRG-mediated increase in FAK phosphorylation at Tyr-861 was blocked by pretreatment of cells with the Src inhibitor PP2 (Fig. 2C). To mechanistically link HRG-mediated Src Tyr-215 phosphorylation to FAK Tyr-861 phosphorylation, we generated a c-Src construct in which tyrosine of Src at 215 was mutated to phenylalanine (Y215F). Using this mutant we analyzed HRG-mediated signaling to FAK at Tyr-861. The results suggest that expression of wild type Src substantially increased both basal and HRG-mediated phosphorylation of FAK at Tyr-861 while expression of the Src Y215F mutant failed to increase HRG-mediated upregulation of phosphorylation of FAK at Tyr-861 (Fig. 2D). Confocal microscopy analysis also showed that HRG induced phosphorylation of FAK Tyr-861 (Fig. 2E, green color, middle panel) as compared to control (Fig. 2E, left panel). Furthermore, HRG-induced ruffle formation as well as FAK Tyr-861 phosphorylation was blocked by pretreatment of cells with the Src-specific inhibitor PP2 (Fig.

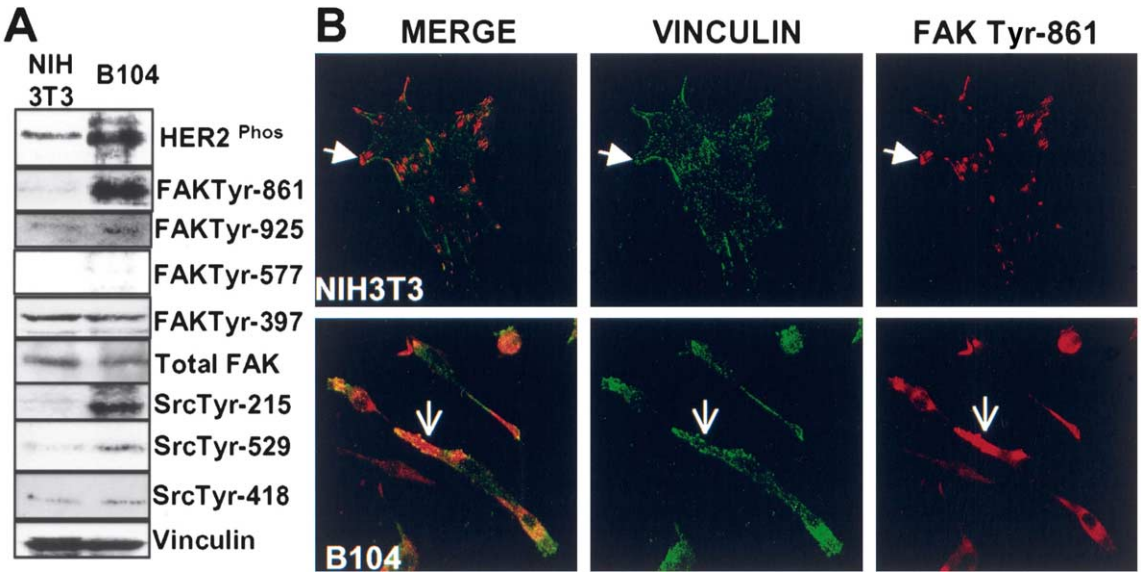

Fig. 3. Analysis of signaling pathways in NIH 3T3 cells expressing constitutively activated HER2. A: Western blot analysis. NIH 3T3 cells and B104 cells (NIH 3T3 cells expressing activated HER2) [13] were lysed and analyzed using phospho-specific antibodies against the indicated molecules. B: Confocal microscopy analysis. The status and localization of FAK Tyr-861 was studied in NIH 3T3 cells and B104 cells. FAK Tyr-861 is shown in red and vinculin, a marker of focal adhesions, is shown in green. Thick arrows point to localization of FAK Tyr-861 in dot-like focal adhesions in NIH 3T3 cells. Empty arrows point to the cytoplasmic localization of FAK Tyr-861.

2E, right panel) [20]. Together these results suggest that HRG-induced stimulation of FAK Tyr-861 phosphorylation might be mediated by c-Src kinase via its activation by phosphorylation at Tyr-215.

3.3. *Activated HER2 modulates phosphorylation of Src at Tyr-215 and FAK at Tyr-861*

We next analyzed the effect of HER2 overexpression on the status of Src Tyr-215 and FAK Tyr-861 using the NIH 3T3 cell line and a well-characterized NIH 3T3 cell line, B104, that expresses activated HER2 [13]. B104 cells exhibited elevated phosphorylation of c-Src Tyr-215 and FAK Tyr-861 compared to the phosphorylation in control NIH 3T3 cells. Minimal or no change was found in the status of tyrosine of FAK at Tyr-577, Tyr-925 and Tyr-397. To confirm the presence and subcellular localization of FAK Tyr-861 phosphorylation in NIH 3T3 and B104 cells, we performed immunofluorescence studies followed by confocal microscopy. The results showed that despite a high level of FAK Tyr-861 phosphorylation in B104 cells, its location was primarily in the cytoplasm (Fig. 3B, thin arrows), as compared to a lower overall amount of phosphorylated FAK Tyr-861 distributed in a distinct dot-like pattern in the parental NIH 3T3 cells (Fig. 3B, thick arrows). Moreover, distinct differences in the cell surface morphology were also noticed (very well spread in NIH 3T3 cells vs. bipolar in B104 cells) and the amount of FAK Tyr-861 per cell surface unit was much greater in B104 cells than in parental NIH 3T3 cells. These findings suggest that active HER2 signaling mimics the events of HRG signaling including phosphorylation of c-Src Tyr-215 and FAK Tyr-861.

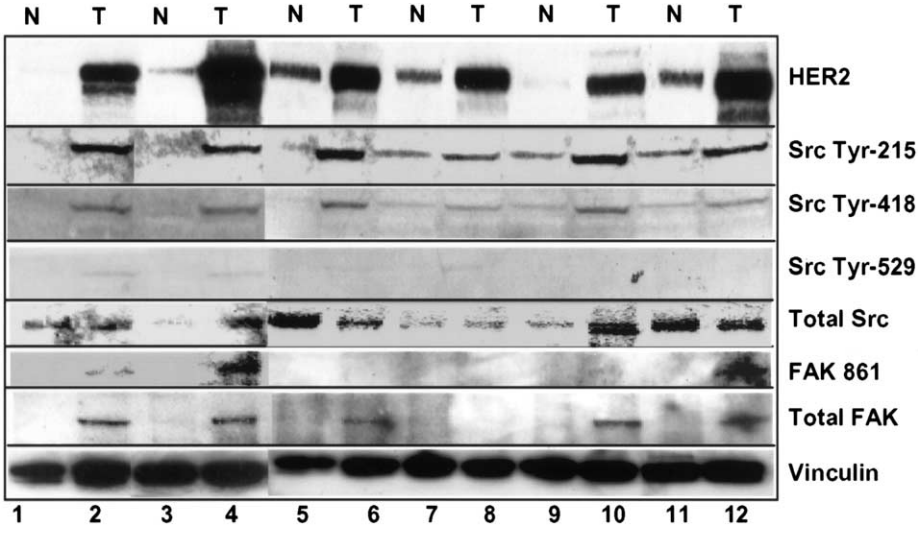

Fig. 4. Upregulation of tyrosine phosphorylation of Src at Tyr-215 in HER2-overexpressing breast tumors. Western blot analysis. Lysates from normal and breast tumor specimens were analyzed by immunoblotting with site-specific antibodies that recognize distinct Tyr sites on Src and FAK kinases. Blots were probed with anti-HER2 antibody to analyze the status of HER2 in tumors. Vinculin was used as a loading control.

3.4. Upregulation of Src Tyr-215 phosphorylation in breast tumors

To confirm the in vitro cell culture data, we analyzed the status of tyrosine phosphorylation of c-Src in a small number of breast tumor specimens. Of the six pairs of breast tumor samples analyzed, five tumors with high HER2 levels exhibited increased levels of tyrosine phosphorylation on Src Tyr-215 compared to tumors without HER2 overexpression or normal controls (Fig. 4). A modest increase in the level of Tyr-418 was also seen in tumors while little change in the Tyr-529 was observed. Results show the coexistence of increased levels of phosphorylation of Src at Tyr-215 and FAK at Tyr-861 in three of six HER2-overexpressing tumors (Fig. 4). These results provide proof of principle that the Src Tyr-215–FAK Tyr-861 pathway we identified in the MCF-7 cell culture model also occurs in breast tumors.

4. Discussion

Src kinase activity has previously been shown to be elevated in HER2-induced mammary tumors [21]. Our results suggest that Src kinase signaling might play a unique role in signaling by HRG and HER2. HRG activated c-Src via tyrosine phosphorylation of Tyr-215 located in the SH2 domain in a dose- and time-dependent manner and increased Src kinase activity. Furthermore, overexpression of constitutively active HER2 also induced phosphorylation of c-Src Tyr-215. Activated c-Src Tyr-215 distinctly localized to actin-containing motile structures such as ruffles in HRG-treated MCF-7 cells and in B104 cells which express activated HER2. In the literature, we could find only one other report showing phosphorylation of Src Tyr-215 by a growth factor, platelet-derived growth factor, and such phosphorylation was reported to result in activation of Src kinase due to disruption of SH2 binding to the C-terminal region of c-Src [22]. Our results support the earlier observation of tyrosine phosphorylation of c-Src at Tyr-215 by growth factors and further suggest that c-Src Tyr-215 phosphorylation may have a unique effect on the localization of c-Src and its substrate specificity.

Despite an enhancement of the c-Src kinase activity by Tyr phosphorylation at Tyr-215 by HRG, only selective change in the phosphorylation of the Src substrate FAK at Tyr-861 was observed. Earlier studies demonstrated that FAK Tyr-861 is a major site of phosphorylation by Src kinase [23]. Vascular endothelial growth factor signaling-mediated endothelial cell migration and anti-apoptosis functions were shown to involve Tyr phosphorylation of FAK at 861 via Src kinase [18]. Recently, phosphorylation of FAK at Tyr-861 was shown to correlate with Ras-induced transformation of fibroblasts [9]. The results of this study suggest that FAK phosphorylation at Tyr-861 also represents a mechanism by which both HRG and activated HER2 may influence the malignant phenotype of epithelial cells since blockage of Src kinase activity by a selective inhibitor PP2 resulted in the reduction of ruffle formation induced by HRG. The findings that HRG and HER2 activated Src kinase and that the Src inhibitor PP2 blocked both the upregulation of FAK Tyr-861 phosphorylation and HRG-mediated cytoskeletal changes suggest that the Src Tyr-215 to FAK Tyr-861 pathway may be important for HRG and HER2 signaling to mediate cytoskeletal changes.

c-Src is also known to utilize Tyr-397, an autophosphorylation site, to associate with FAK [6]. Since HRG did not cause any changes in the phosphorylation of FAK Tyr-397, HRG- and HER2-activated c-Src may interact with FAK via other interacting sites, and selectively phosphorylate c-Src at Tyr-861. In this context, v-Src was previously shown to induce FAK Tyr phosphorylation, independently of Tyr-397 phosphorylation [24]. Identification of HRG signaling to FAK kinase via phosphorylation of the SH2 domain raises the possibility that the phospho antibodies against c-Src Tyr-215 may serve as a prognostic marker. However, a larger study would need to be conducted to establish the significance of these tools. In summary, our results suggest that HER2 and HRG systems uniquely regulate signaling from focal adhesion complexes through selective phosphorylation of c-Src Tyr-215 and FAK Tyr-861.

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