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PRINCIPAL INVESTIGATOR: Allison P. Belsches
Sarah J. Parsons

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, Virginia 22908

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6. AUTHOR(S)
Allison P. Belches
Sarah J. Parsons

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Virginia
Charlottesville, VA 22908

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To investigate whether interactions between c-Src and other HER family members may play a role in breast tumor progression, we characterized a panel of thirteen human breast carcinoma cell lines and thirteen tumor samples for expression levels of HER family members and c-Src, and examined a subset of the cell lines for their dependency on Src family members for heregulin (HRG)-augmented, anchorage-dependent growth in reduced serum or colony formation in soft agar. We found that 90-100% of the samples overexpressed one or more HER family members and that c-Src was overexpressed in approximately 70% of the cases. Growth in low serum was potentiated by HRG in five of six cell lines tested (irrespective of HER2/3 or c-Src levels), and colony formation in soft agar was enhanced in only those cell lines that exhibited a c-Src/HER2 heterocomplex, suggesting that physical association between c-Src and HER2 may facilitate HRG-potentiated, anchorage-independent growth. HRG effects were either partially or completely ablated by PP1, a Src family kinase inhibitor. In addition, long-term treatment of adherent cells with PP1 induced apoptosis in all cell lines tested, a process that was significantly reversed by HRG treatment. These data provide the first functional evidence for co-operativity between HRG and Src family kinases in the survival and growth of human breast tumor cells.

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FOREWORD

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Michael P. Hobbell 7/26/99

PI Signature Date

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INTRODUCTION

Recently, several laboratories have reported that functional interactions between members of the HER receptor tyrosine kinase receptor family and c-Src, a non-receptor tyrosine kinase, may play a role in breast tumor formation and progression. To determine if c-Src could functionally interact with HER2 as it does with HER1, one approach was to examine a panel of human breast tumor cell lines and tumor tissues for levels of HER family members and c-Src and for evidence of physical and functional interactions between HER2 and c-Src. A second approach to test possible functional interactions between HER2 and c-Src was to overexpress HER2 and c-Src, either alone or together, in C3H10T1/2 murine fibroblasts as a model system. The progress made towards the specific aims of this grant proposal is discussed in Sections A and B of the Body of this final report. In addition to investigating the Specific Aims identified in the grant, our research has led us to study some additional topics that are described in Section C of the Body of the final report.

BODY

A) Summary of the Progress in Accomplishing Specific Aims II and III.

The accomplishment of Specific Aims II and III is summarized in the manuscript entitled "Src Family Kinases and Heregulin Cooperate to Promote Human Breast Cancer Cell Growth and Survival," which was submitted to Cancer Research. This manuscript is located in the Appendix of this final report.

B) Summary of Successes and Difficulties in Accomplishing Specific Aims I and IV.

In order to understand the potential physical and functional interaction between HER2/*neu* and c-Src in the progression of human breast cancer, we have proposed to study HER2/*neu* and c-Src in the C3H10T1/2 murine fibroblast cell line. In the past, we have successfully used the C3H10T1/2 model system to investigate the physical and functional interaction between HER1 and c-Src in human breast cancer. These studies have culminated in the finding that HER1 is a substrate of c-Src and that one of the sites of phosphorylation on the receptor (Y845) is required for EGF-dependent mitogenesis. In order to understand the biological function of HER2/*neu* and c-Src interaction, we are developing similar cell lines that overexpress wild-type HER2/*neu* and c-Src alone or in conjunction with one another. In characterizing the C3H10T1/2 cell line by Western immunoblotting, we were not able to detect murine erbB3 (HER3) or erbB4 (HER4), the heterodimerization partners of HER2. Furthermore, we found that overexpression of HER2/*neu* alone in the C3H10T1/2 cell line resulted in cell death. Cells that survived transfection with HER2 under the control of a constitutive mammalian promoter or a murine sarcoma virus LTR and antibiotic selection did not overexpress the exogenous HER2 and were found to express a protein smaller than the 185 kDA protein band expected for HER2. This suggests that HER2 overexpression alone is apoptotic and surviving cells were able to downregulate expression of the exogenous gene product

either by preventing post-translation modification or through endocytosis and degradation. In an attempt to overcome HER2-induced apoptosis, we are 1) investigating the possibility that co-overexpression of c-Src and/or HER3 with HER2 will promote survival and induce an oncogenic phenotype, and 2) also using MCF10A cells and MCF7 as recipients of HER2/c-Src/HER3 to determine if cell type or genetic background are important for stable expression of HER2 as well as for cell survival and/or transformation. It is hypothesized that HER2 may interact with c-Src in order to promote cell survival. Likewise, HER3 is not only a dimerization partner for HER2 but also a strong recruiter of PI3-kinase, a known survival factor. Others have demonstrated that HER2/HER3 is a preferred heterodimerization partner in the presence of heregulin and that this complex results in strong kinase activity. It is hypothesized in C3H10T1/2 cells, that co-overexpression of HER3 and/or c-Src with HER2 will result in stable overexpression of HER2, promotion of cell survival, and transformation. If the hypothesis is true, it would provide the first explanations for the observations that HER2 is frequently co-overexpressed with HER3 and c-Src is frequently complexed with HER2 in human breast cancers. Both of these explanations can be tested by further experimentation, using the stable transfectants of variants of HER2 in 10T1/2 cells that we have already generated (Table I). These include kinase defective HER2 (L753R) and HER2 mutated at Y877 (the homologue of Y845 in HER1 that is phosphorylated by c-Src). These cell lines will be analyzed for growth and tumorigenesis along with the wild type HER2, HER3/ or c-Src double overexpressors as described in Aims I and IV.

C) Related Studies.

In addition to the specific aims outlined in this grant proposal, several related studies were performed to investigate the role of another c-Src substrate, p190RhoGAP (190 kD, GTPase-activating protein for Rho) in cell growth. p190RhoGAP was first identified as a tyrosine phosphorylated protein that was associated with p120RasGAP (GTPase-activating protein for Ras), suggesting that p190 may play a role in regulating signaling through Ras, ultimately effecting mitogenesis [1]. Upon cloning, p190RhoGAP was shown to have two functional domains, an amino-terminal GTP-binding domain and a carboxyl-terminal GTPase-activating protein domain with specificity for Rho family members [2,3] (see Figure 1). Rho is active when bound to GTP and causes actin stress fiber formation, whereas Rho in the GDP-bound form causes actin stress fiber disassembly [4]. The finding that p190 is a GAP for Rho suggests a link between p190 and the actin cytoskeleton. Changes in the actin cytoskeleton have been linked to cellular transformation as well as tumor formation [5].

Our laboratory has demonstrated that p190 is a substrate of c-Src, and that c-Src preferentially phosphorylates p190 on tyrosine 1105 which resides in the middle domain of p190 [6-8]. Furthermore, we have also demonstrated that in serum starved C3H10T1/2 cells, p190 is diffusely localized throughout the cytoplasm, and upon EGF stimulation, p190 transiently translocates to arc-like structures that radiate away from the nucleus. The time course of localization of p190 into the arcs is variable in cell lines that overexpress wild type c-Src , kinase inactive c-Src, or control cells suggesting that c-Src phosphorylation of p190 may regulate its activity. In neo control cells, p190 is located in

the arc-like structures at two minutes [7]. Overexpression of c-Src causes an increase in the kinetics of p190 localization into the arc-like structures occuring at 30 sec after EGF stimulation. In contrast, the localization of p190 in cells that overexpress kinase inactive c-Src is delayed as compared to the neo control cells (5 min versus 2 min) [7]. The time course of EGF-induced actin stress fiber disassembly coincides with the localization of p190 into the arc-like structures in each of the cells types. Taken together, these observations suggest that p190 may play a role in EGF-induced actin stress fiber disassembly and that c-Src regulates p190 function through p190 tyrosine phosphorylation.

To identify which domains of p190 are required for regulation of the actin cytoskeleton, we generated six GST bacterial fusion proteins of p190: wild type (*wt*) GAP domain (aa1261-1469), Y1283F and Y1283D mutants of the GAP domain, *wt* middle domain (aa380-1180), *wt* middle domain/GAP domain (aa380-1469), and *wt* section 3 of the middle domain construct (aa919-1180). These proteins were purified, microinjected into C3H10T1/2 murine fibroblasts, and then stimulated with EGF over a 30 min time course. Our data show that microinjection of *wt* GAP domain causes a dramatic, EGF-independent disassembly of actin stress fibers in these cells (Figures 2 and 3). However, when either the Y1283F or Y1283D mutants were injected, no actin dissolution was observed above the control GST-injected levels (Figure 4). *In vitro,* Rho GTPase assays demonstrated that the GAP activity of the mutants was severely diminished when compared to the GAP activity of *wt* RhoGAP domain (data not shown). Together, these data indicate that the p190 RhoGAP activity is required for actin stress fiber disassembly. Furthermore, injection of the middle domain/GAP construct in Neo cells restored EGF regulation of the RhoGAP activity and partially reduced the extent of actin dissolution seen in normal controls (Figures 2 and 3). *In vitro,* Rho GTPase assays demonstrated that GAP activity of the GST-GAP and the GST-MD/GAP were equivalent (Fig. 5). These results indicate that the middle domain may negatively regulate the activity of the GAP domain, and thus the ability of p190 to regulate cytoskeletal changes.

In addition to regulating the actin cytoskeleton, the Rho family of small GTP-binding proteins function in gene transcription, cell cycle regulation, apoptosis, and tumor progression [9]. Therefore, p190, by regulating Rho, may be involved in these processes. We hypothesized that by identifying binding proteins for p190, we would gain insight into the function of p190. We have focused on identifying proteins that interact with the middle domain of p190 because in addition to containing Y1105 (that when phosphorylated can bind to SH2 domains of proteins, such as p120RasGAP), the middle domain contains five PXXP motifs that bind to SH3 domains. Using the yeast two-hybrid system, I isolated thirty-one clones that specifically bound to Section 1 of the p190 middle domain (aa 380-645, see Figure 1 in the appendix). A list of some of these p190 interacting proteins is located in Table II. Interestingly, many of the identified p190-binding proteins, including cytochrome C, survivin, neuroserpin, histone deacetylase, and glyceraldehyde 3 phosphate dehydrogenase are known to play a role in apoptosis, which suggests that p190 may be a regulator of growth. I am currently investigating the binding of p190 to these apoptotic proteins by co-immunoprecipitation and co-immunofluorescence studies. Interestingly, the finding that histone deacetylase also binds to p190 may indicate a role for p190 in gene transcription or cell cycle progression.

Consistent with the findings from the yeast two-hybrid system, recent results from our laboratory have demonstrated that transient overexpression of p190RhoGAP in C3H10T1/2 murine fibroblasts induces cell death (nuclear condensation)(unpublished observation). We have determined that the GTPase domain and the middle domain of p190 are necessary for this cell death and that this cell death is independent of the ability of p190 to bind GTP. However, the GAP domain of p190 is not required for cell death, which may indicate a Rho-independent function for p190. These data suggest that p190 may function as a tumor suppressor, and perhaps is regulated by binding to the proteins that are listed in Table II (manuscript in preparation). Because p190 is a substrate of c-Src, it is conceivable that tyrosine phosphorylation of p190 may also regulate its ability to act as a negative regulator of growth. Therefore, we are overexpressing p190 in C3H10T1/2 fibroblasts that overexpress c-Src to see if p190-induced cell death is abrogated by c-Src. To directly test the hypothesis that p190 can function as a tumor suppressor, we are currently screening the panel of human breast carcinoma cell lines to determine the expression levels and extent of tyrosine phosphorylation of p190. We are also attempting to transiently and stably transfect p190 into a series of breast cancer cell lines, including MDA-MB 468, MDA-MB 361, MCF7, MDA-MB231, and MDA-MB 453 to determine what effect p190 has on DNA synthesis as a measure of cell growth. We hope to be able to use these data to determine if there is a correlation between c-Src phosphorylation of p190, expression levels of p190, and cell growth. The results of these experiments may indicate that c-Src and p190 act as regulators of breast tumor growth, and may lead to novel therapies for treating breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Our work has shown that HER family members as well as c-Src are overexpressed in the majority of human breast tumor cell lines and breast tumors tested (total of 27).

- In 3 of 14 breast tumor cell lines and in 3 of 13 human breast tumors, HER2 and c-Src are physically associated in an immune complex. However, in contrast to the HER1/Src model system, overexpression of neither HER2 nor c-Src is required for physical association.

- Only the three breast tumor cell lines exhibiting the HER2/c-Src complex (MDA-MB-361, MDA-MB-453 and UACC-812) show an increase in number of colonies formed in soft agar in the presence of HRG. This effect was ablated by the Src family kinase inhibitor, PP1. These data suggest a correlation between HER2/c-Src association and HRG-dependent increased anchorage-independent growth.

- In low serum, anchorage-dependent growth assays (MTT) of six breast tumor cell lines tested, modest and variable growth responses to HRG were observed all the cell lines. However, PP1 inhibited HRG-augmented growth of all but one cell line and also inhibited HRG-independent growth in the majority of cell lines. These data suggest

that Src family kinases participate in HRG-dependent, as well as HRG-independent adherent cell growth.

- Overexpression of wild type HER2 in C3H10T1/2 cells results in apoptotic cell death.
- The p190 middle domain negatively regulates the activity of the p190 GAP domain.
- Using the yeast two-hybrid system, cytochrome C, survivin, neuroserpin, histone deacetylase, and glyceraldehyde 3 phosphate dehydrogenase have been identified as p190-binding proteins. All of these p190-interacting proteins have been shown to participate in cell death (apoptosis).
- Transient overexpression of p190 induces cell death in C3H10T1/2 murine fibroblasts.
- The p190 GTPase domain and the middle domain are necessary for the induction of cell death, whereas, the p190 RhoGAP domain is not required for p190-induced cell death. These data suggest that p190 functions independently of Rho in inducing cell death.

REPORTABLE OUTCOMES

- **Manuscripts:** The manuscript entitled "Src Family Kinases and Heregulin Cooperate to Promote Human Breast Cancer Cell Growth and Survival," was submitted to Cancer Research.

- **Abstracts:** A list of the meetings attended and the abstracts submitted during the funding period of this grant is located in the Final Report Section (pg. 59-64)

- **Presentations:** Poster presentations- Keystone, 1997; DOD, 1997; Oncogene, 1997; Cold Spring Harbor, 1998; Keystone, 1999; and Oncogene, 1999.
  Oral presentation- Oncogene, 1999

REPORTABLE OUTCOMES (cont.)

- **Development of Cell Lines:**

Table I
Development of stably transfected cell lines.

Constructs Needed:	Control Background:	c-Src Overexpressor:
Wild type HER2		
Oncogenic HER2 (V659E)	4 (5X)[1]	
HER1/HER2 chimera		
Kinase-deficient HER2 (L753R)		3 (24X)
Phosphorylation-site mutant HER2 (Y877F)	3 (6X)	

[1] Indicates number of clones obtained and (fold overexpression).

Table I. Development of stably transfected cell lines. Stable clones of C3H10T1/2 mouse fibroblasts which overexpress wildtype or mutant HER2 protein are listed, with the relative levels of expression above parental 10T1/2 cells shown in parentheses. c-Src overexpressors cells (5Hd47) express c-Src protein approximately 25-fold over endogenous 10T1/2 levels (Luttrell *et al.*, 1988, Wilson, *et al.*, 1989).

- **Funding Applied For:** 7/23/99, submitted an NIH P20 RFA on cellular and small animal imaging in cancer. Our project proposes to use HER1, HER2, and c-Src transgenic mice as animal models for breast tumor development. M. Williams, M.D., P.I.

CONCLUSIONS

This work provides the first demonstration of a functional relationship between HER2 and c-Src that correlates with a physical association between the two molecules.

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APPENDICES

Table I
Development of stably transfected cell lines.

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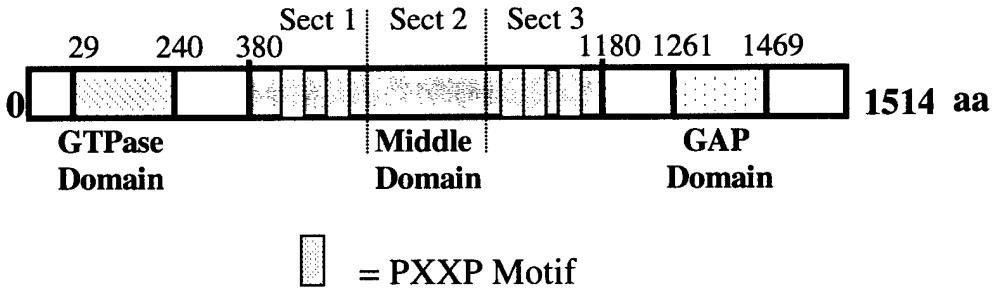

Figure 1. Schematic Figure of p190RhoGAP. The GTPase domain consists of amino acids 29-240, the middle domain consists of amino acids 380-1180, and the GAP domain consists of amino acids 1261-1469. The middle domain contains five PXXP motifs (indicated in the hatched boxes) as well as tyrosine 1105.

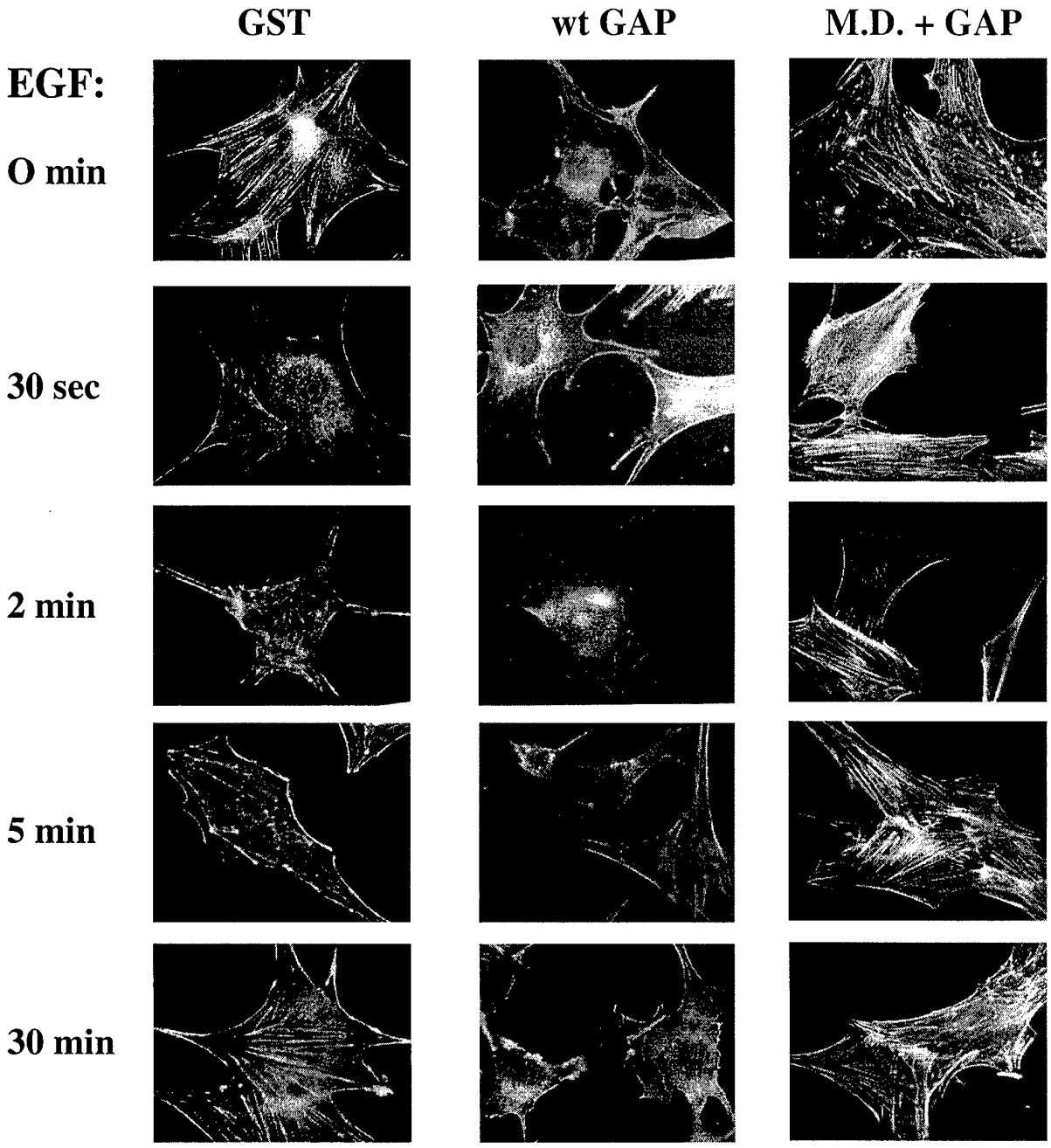

Figure 2. FITC-Phalloidin staining of actin stress fibers in neo control cells microinjected with GST, GST-p190 GAP domain, GST-p190 middle domain/GAP and stimulated with EGF. Serum-starved Neo control cells (C3H10T1/2) were microinjected (at least 70 cells per coverslip)with equimolar concentrations of GST (0.5 mg/ml), GST-p190 GAP domain (0.5 mg/ml), or GST-p190 middle domain/GAP (2.2 mg/ml), and after a 15 min recovery period, the cells were stimulated with 100 ng/ml EGF at 37° C for the indicated times (0-30 min). The actin stress fibers were stained with fluorescein-conjugated phalloidin. All of the cells shown have been injected.

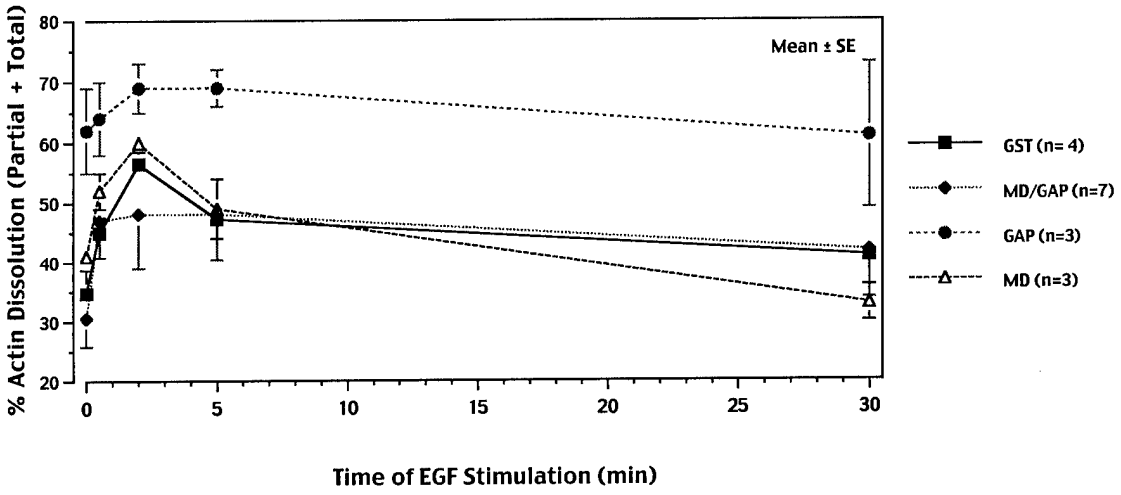

Figure 3. Microinjection of the GST-p190 middle domain/GAP construct inhibits the constitutive actin stress fiber disassembly induced by the p190 GAP domain. Serum-starved neo control cells were microinjected with the GST-p190 fusion protein constructs and stimulated with 100 ng/ml EGF as described in Fig. 2. The percent of cells displaying actin stress fiber disassembly was calculated. Cells were scored positive for actin stress fiber disassembly if greater than 50% of the actin stress fibers had been disrupted. At least 50 cells were counted per coverslip.

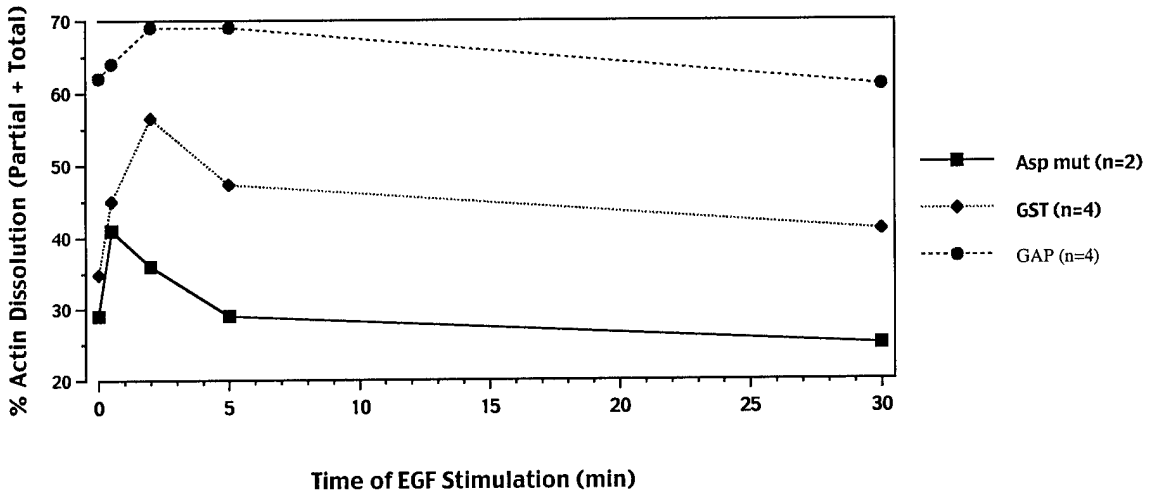

Figure 4. The GST-p190 GAP mutant (Y1283D) does not induce the constitutive actin stress fiber disassemby observed with wild type GST-p190 GAP and has a dominant negative effect on EGF-induced actin stress fiber assembly. Serum-starved neo control cells were microinjected with the wild type GST-p190 GAP (0.5 mg/ml), or mutant GST-p190 GAP (Y1283D), or GST (0.5 mg/ml) and stimulated with 100 ng/ml EGF as described in Fig. 2. The percent of cells displaying actin stress fiber disassembly was calculated. Cells were scored positive for actin stress fiber disassembly if greater than 50% of the actin stress fibers had been disrupted. At least 50 cells were counted per coverslip.

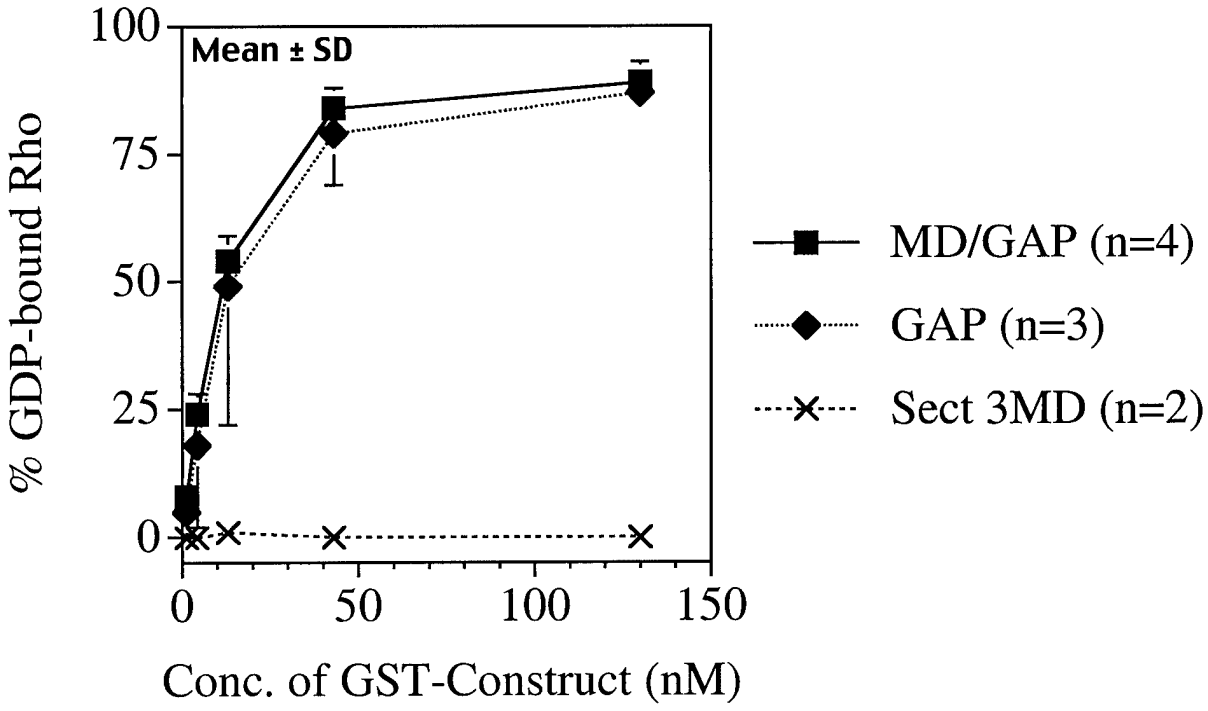

Figure 5. The GST-GAP and GST middle domain/GAP constructs have similar RhoGAP activity *in vitro*. Various concentrations of GST-GAP and GST-middle domain/GAP (1.3-130 nM) were incubated with 250 ng of [γ^{32}P]GTP-RhoA for 10 min at RT. The reactions were stopped by the addition of ice cold stop buffer. The RhoA was collected by vacuum filtration onto nitrocellulose filters and the percent of hydrolysis of [γ^{32}P]GTP-RhoA was determined.

Clone #	Protein Match	# of Hits
3	Survivin	1
23	Cytochrome C	1
14	Neuroserpin	1
4	M phase phosphoprotein	1
26, 27, 19, 20	Histone deacetylase	4
11, 28	Glyceraldehyde 3 phosphate dehydrogenase	2
31	p190RhoGAP (GTP-binding domain)	1
18	Alpha adrenergic receptor	1
17	Platelet-derived growth factor receptor	1

Table II. p190-binding proteins identified using the yeast two-hybrid system.

Submitted
2/22/97
Cancer Research

Src Family Kinases and Heregulin Cooperate to Promote Human Breast Cancer Cell Growth and Survival

Allison P. Belsches-Jablonski, Jacqueline S. Biscardi, Dena R. Peavy, David A. Tice, Davis A. Romney, and Sarah J. Parsons*

Department of Microbiology and Cancer Center, Box 441, University of Virginia Health Sciences Center, Charlottesville, VA 22908

Running Title: c-Src interaction with HER2/*neu* in Human Breast Cancer

*Corresponding author

TEL: 804-924-2352

FAX: 804-982-0689

email: sap@virginia.edu

Key words: Src family kinases
Heregulin
Breast cancer
HER2/*neu*
Apoptosis

ABSTRACT

Evidence from a murine fibroblast model and human breast cancer cells indicates that c-Src and the human EGF receptor (HER1) synergize to enhance neoplastic transformation and progression of mammary epithelial cells. To investigate whether interactions between c-Src and other HER family members may also play a role in breast tumor progression, we characterized a panel of thirteen human breast carcinoma cell lines and thirteen tumor samples for expression levels of HER family members and c-Src and examined a subset of the cell lines for their dependency on Src family members for heregulin (HRG)-augmented, anchorage-dependent growth in reduced serum or colony formation in soft agar. By Western immunoblotting, we found that (1) all cell lines overexpressed one or more HER family member, (2) ~60% co-overexpressed HER2/*neu* and HER3, (3) HER1 was rarely co-overexpressed with HER2/HER3, and (4) HER4 was overexpressed in only 30% of the cases. c-Src was overexpressed in 70% of the samples. 90% of the tumor tissues overexpressed HER2, while 64% overexpressed c-Src. Growth in low serum was potentiated by HRG in five of six cell lines tested (irrespective of HER2/3 or c-Src levels), and colony formation in soft agar was enhanced in three of the six. This latter response was observed only in those cell lines that exhibited a c-Src/HER2 heterocomplex, suggesting that physical association between c-Src and HER2 may facilitate HRG-potentiated, anchorage-independent growth. HRG effects on both colony formation and growth in reduced serum were either partially or completely ablated by PP1, a Src family kinase inhibitor. In addition, long-term treatment of adherent cells with PP1 induced apoptosis in all cell lines tested, a process that was significantly reversed by HRG treatment. These data provide the first functional evidence for co-operativity between HRG and Src family kinases in the survival and growth of human breast

tumor cells and reveal that the co-operativity is independent of the levels of either HER family members or c-Src.

INTRODUCTION

The HER family of transmembrane tyrosine kinases consists of four members, the human EGF receptor (EGFR or HER1), HER2/*neu,* HER3 and HER4. These receptors are involved in regulation of a number of different cellular processes, including mitogenesis, tumorigenesis, and differentiation (1, 2). HER1 has been shown to bind and become activated by different members of the EGF family of ligands, including EGF, TGF-α, epiregulin and betacellulin (3-8). HER4 binds betacellulin (5) and epiregulin (7), and like HER3, functions as a receptor for neuregulin 1 (also known as heregulin [HRG] and neu differentiation factor) and neuregulin 2 (9-11). However, no ligand has been identified for HER2. Rather, it has been demonstrated that HER2 is activated through heterodimerization with ligand-activated HER1, HER3 or HER4, with HER2/3 being the preferred and most tumorigenic heterodimer (12-15). Dimerization is followed by transphosphorylation between paired receptor monomers and stimulation or recruitment to the receptor complex of signaling molecules, including PI-3 kinase (phosphatidylinositol-3 kinase), Shc, GRB2, and GRB7 (16-19). In the HER2/3 heterodimer, HER3 (which exhibits reduced intrinsic catalytic activity [20]) appears to provide ligand binding activity and the ability to associate with PI-3 kinase, while HER2 contributes kinase activity and the ability to phosphorylate downstream substrates as well as the ability to associate with PI-3 kinase (9, 21, 22).

HER2 has potent oncogenic activity in cultured fibroblasts when overexpressed as a wild type protein, or when activated by a single point mutation in the transmembrane domain or by

deletion of the first 621 amino acids from the N-terminus (human [23]; rat [24]). Increased expression of HER2 is observed in a number of human cancers, including breast (25), ovarian (26), gastric (27), lung (28) and prostate (29). In breast cancers, overexpression and/or amplification of HER2, which occurs in ~ 30 percent of the cases, has been correlated with poor prognosis (25, 30). Overexpression of HER2 can function as a causal factor for breast cancer development, as has been demonstrated by targeted overexpression of HER2/*neu* and development of mammary tumors in mice (31).

Recently, several laboratories have reported the detection of elevated levels of c-Src protein/activity or physical complexes between members of the HER receptor tyrosine kinase family and c-Src in human and mouse mammary tumors (32-39). These findings suggest that a functional interaction occurs between these two families of tyrosine kinases in tumor formation and progression, but only in the case of HER1 and c-Src has such biological synergism been demonstrated. In a murine fibroblast model, overexpression of c-Src was found to potentiate tumorigenesis induced by overexpression of HER1. This potentiation correlated with the EGF-inducible formation of a stable complex between c-Src and HER1, the appearance of two novel tyrosine phosphorylation sites on the receptor, and enhanced phosphorylation of receptor substrates, suggesting that when both c-Src and HER1 are overexpressed (as in human breast cancer cells), c-Src functionally synergizes with the receptor by phosphorylating and activating it (36, 40, 41). The relevance of this model for human neoplasias is supported by an analysis of panels of human breast cancer cell lines and tumor tissues, in which overexpression of both c-Src and HER1 was found to correlate with the ability to detect a stable heterocomplex between c-Src and HER1, the appearance of the same two novel phosphorylations on the receptor, elevated tyrosine phosphorylation of downstream targets of HER1, and enhanced tumor formation in nude

mice (40). Together, these results indicate that structural and functional interactions between c-Src and HER1 play critical roles in breast cancer tumor progression.

To determine whether c-Src is capable of functionally interacting with other HER family members as it does with HER1, a panel of human breast tumor cell lines and tumor tissues was first examined for levels of HER family members and c-Src and for evidence of physical interactions between HER2 and c-Src. Using a subset of these lines (selected for various patterns of HER family member and c-Src overexpression and HER2/c-Src association), HRG was used as a ligand to activate HER2 through HER3 or HER4. We observed that HRG increased colony formation in soft agar two to three fold above that in serum alone and that this enhanced anchorage-independent growth correlated with the ability to detect stable c-Src/HER2 complexes. Anchorage-dependent growth in low serum was also enhanced by HRG, but to varying extents among the six cell lines tested. No correlation was observed between levels of c-Src or HER 2/3/4 and the ability of HRG to enhance growth. However, in all cell lines, HRG-enhanced growth in reduced serum and soft agar colony formation were partially or completely inhibited by PP1, a Src family kinase inhibitor (42). Interestingly, extended PP1 treatment was found to induce apoptosis, and HRG rescued this effect. Taken together, these data provide evidence for co-operation between c-Src and HER2 in mediating HRG-induced growth and survival of human breast tumor cells.

MATERIALS AND METHODS

Cell lines. Tumor cell lines MDA-MB-468, MDA-MB-231, MCF7 and ZR75-1 were provided by N. Rosen (Sloan-Kettering, New York), while MDA-MB-175, UACC-893, UACC-812, SK-BR-3, MDA-MB-361, MDA-MB-453, BT-474, BT-549, BT-20 and HS578Bst were obtained

from American Type Culture Collection (Rockville, MD). Tumor cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Bethesda, MD) in 10% fetal calf serum (Atlanta Biologicals, Atlanta, GA). Where indicated, cells were stimulated with recombinant human HRG-α, EGF-like domain (HRG-α) (200ng/ml) (Sigma Chemical, St. Louis, MO) or HRG-β1 (extracellular domain) (NeoMarkers, CA). HRG-α and HRG-β1 gave nearly identical results and were used interchangeably.

Growth assays. Tumor cell lines were plated in triplicate in DMEM plus 0.5% fetal calf serum at a density of either $2x10^4$ cells/well (MDA-MB-468, MCF7) or $5x10^4$ cells/well (UACC-812, SK-BR-3, MDA-MB-361, MDA-MB-453) in 24-well tissue culture clusters (Corning, NY) and allowed to adhere overnight at 37^0C in a humidified, 5% CO_2 atmosphere before treatment protocols were initiated. Cells were then incubated for five days with one of the indicated preparations in DMEM. Media with additives was replenished on the third day. Cell number was determined by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay as per Kaspers, *et al.* (43). The Src family kinase inhibitor, PP1 (42), was obtained from CalBiochem, San Diego, CA.

Soft agar assays were performed as described by Maa, *et al.* (36). Cells were plated at a density of $1x10^4$ cells in 60 mm dishes in triplicate, in the presence or absence of HRG-α (40ng/ml), and in the presence or absence of PP1 (10 μM). In the absence of PP1, DMSO was used as a vehicle control. Plates were incubated for two weeks, with replenishment of appropriate media every 4 days, and stained overnight at 37°C, 5% CO_2 in a solution of 1 μg/ml iodonitrotetrazolium salt (Sigma Chemical, St. Louis, MO) in water. Colonies were counted using EagleSight analysis software (Stratagene, La Jolla, CA).

Antibodies. C-Src-specific antibodies used in this study include mouse monoclonal antibodies (mAb) 2-17, directed against amino acids 2-17 (Quality Biotech, Camden, NJ); mAbs GD11 and EB8, both directed against residues 92-128 in the SH3 domain (44, 45); and mAb 327, which recognizes the SH3 domain (gift from J. Brugge). HER2-specific antibody (rabbit polyclonal), directed toward residues 1169-1186, and HER3-specific antibody, directed toward residues 1307-1323, were obtained from Santa Cruz Biotechnologies, Santa Cruz, CA, USA. Antibodies specific for HER1, mAbs 3A and 4A, were provided by D. McCarley and R. Schatzman of Syntex Research, Palo Alto, CA. The epitopes of these antibodies map to amino acid residues 889-944 and 1052 and 1134, respectively (36). Anti-human c-erbB-4 antibodies were obtained from Upstate Biotechnology, Inc. (UBI), Lake Placid, NY (catalog #06-572) and Santa Cruz Biotechnologies (catalog #SC-283). Negative control antibodies included purified normal rabbit or mouse immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, PA, USA).

Immunoprecipitation and Western immunoblotting. Cells were lysed in RIPA detergent buffer (10mM Tris-HCl pH 7.2, 1% Triton-X, 0.5% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM sodium orthovanadate, 50 μg/ml leupeptin and 0.5% aprotinin). Tumor samples were minced with a scalpel and ground in a Dounce homogenizer in RIPA buffer. All procedures were performed at 4-10^{0}C. Lysates were cleared by centrifugation for 15 min at 15,000 x g in a microcentrifuge, and protein concentration was determined by BioRad protein reagent. For immunoprecipitation, 5 μg antibody was incubated with 500 μg cell lysate protein with continuous rocking at 4^{0}C for 2 hr and then for an additional hour at 4°C with protein A-sepharose beads (Sigma Chemical). Beads were pelleted and washed three times with cold RIPA buffer. Pellets were resuspended in 2X sample buffer (125mM Tris-HCl, 4% SDS, 10% glycerol, 0.02% bromophenol blue, 4% β-mercaptoethanol) and boiled for 5 min. Eluted proteins were

separated by electrophoresis through a 7% SDS-polyacrylamide gel and immunoblotted according to previously published protocols (36, 37). For direct Western blots, 100 μg whole-cell lysate was assayed per lane. Binding of primary antibodies was visualized by ^{125}I-protein A, used at 1 μ Ci/ml (New England Nuclear, USA), or by enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, England).

For Western blots of HER4, proteins were transferred from gels to Immobilon membranes using the semi-dry apparatus. Membranes were blocked in phosphate-buffered saline (PBS) containing 4% non-fat dry milk and incubated overnight at 4°C with a mixture of the two primary anti-HER4 antibodies at a final concentration of 2 μg/ml. Membranes were washed five times in deionized water and incubated with ^{125}I-goat anti-rabbit IgG in 4% non-fat dry milk in PBS for 90 min at room temperature. Five washes in water, a single wash in PBS, and five additional washes in water followed. The blot was exposed to film overnight at -70°C.

Apoptosis Assays. Apoptosis was quantified by determining the percentage of cells that exhibited deformed nuclei and/or condensed chromatin following DAPI (di-[amidinophenyl]-indole) staining of treated cells, as described by Villaneuva *et al.* (46). Briefly, cells were plated onto glass coverslips in 6-well dishes (Corning, NY), and incubated for three days with DMEM containing the indicated additives. UV-B treatment (2 min, followed by 48 hr recovery in serum-free media) was used as a positive control for apoptosis. After treatment, cells were serum-starved overnight, fixed in 4% paraformaldehyde, quenched in 0.25mg/ml H_3BO_4 and 20mg/ml glycine, and permeabilized in 0.5% Triton-X on ice. DAPI (1.0 μg/ml) (Sigma Chemical, St. Louis, MO) was added for specific DNA staining, and nuclear/chromatin morphology was imaged by photomicroscopy using phase contrast and immunofluorescence optics (Leica, Rijswijk, Netherlands).

RESULTS

Overexpression of c-Src and HER family members in human breast carcinoma cell lines. A panel of thirteen human breast carcinoma cell lines was analyzed by Western blotting for expression levels of c-Src and HER family members (a representative series of blots is shown in Fig. 1). Results of the scanning densitometry analysis of the immunoblots is shown in Table I. Nine of the cell lines (~70%) expressed c-Src greater than two-fold above the immortalized, non-tumorigenic human breast epithelial cell line, HS578Bst. All of the tumor cell lines overexpressed one or more of the four HER family members, in various combinations. Within any given cell line, however, overexpression of HER1 and HER2 appeared to be mutually exclusive. This finding agrees with published literature, which indicates that HER2 is typically expressed in earlier stage breast carcinomas (47-49), while HER1 is expressed in later, more aggressive tumors (50, 51). In addition, HER2 and HER3 were found to be co-overexpressed in the majority of cell lines (~60%), thereby enhancing the probability of formation of the preferred, more tumorigenic HER2/3 heterodimer. Under our immunoblotting conditions, only two cell lines, ZR75B and BT-474, were observed to have significantly elevated levels of HER4, with low levels being detected in SK-BR3 and MCF7. In summary, 100% of the breast tumor cell lines overexpressed one or more HER family member, while 70% overexpressed c-Src.

Physical association of c-Src with HER2 in human breast carcinoma cell lines. To determine whether there were potential structural and functional interactions between c-Src and HER2, such as exists between c-Src and HER1, we analyzed the panel of human breast carcinoma cell lines for evidence of *in vivo* association between c-Src and HER2. Figure 2, Panel A depicts results from an analysis of four of the thirteen cell lines tested (MDA-MB-361, MDA-MB-453, MCF7, MDA-

MB-468), and the outcome of the entire analysis is summarized in Table I. In only three cell lines of the entire panel could we detect a stable *in vivo* association between c-Src and HER2 (MDA-MB-361, MDA-MB-453, UACC-812), although nine overexpressed HER2 and five of the nine overexpressed c-Src. The presence of HER2 in c-Src immunoprecipitates was reproducibly seen, but detection of c-Src in HER-2 immunoprecipitates was variable. The reason for this is unclear, but could relate to the relatively low stoichiometry of the interaction. Association was constitutive in serum-deprived cells and was not altered by HRG stimulation (200ng/ml for 5 min) (Figure 2, Panel A). Only one of the three cell lines that showed association overexpressed c-Src (MDA-MB-361), indicating that association was not dependent on overexpression of c-Src. However, HER2 and HER3 were overexpressed in all three cell lines.

The low level of c-Src in the MDA-MB-453 and UACC-812 cell lines complicated our efforts to convincingly demonstrate that c-Src was indeed in the HER2/c-Src complex. However, despite the fact that c-Src could not be detected by Western blotting in whole-cell lysates (Fig. 1 and Table I) or in some c-Src-immunoprecipitations using 2-17 c-Src-specific mAb and 500 μg lysate (Fig. 2A with the MDA-MB-453 cell line), Figure 2, Panel B shows that c-Src could be detected in the MDA-MB-453 cell line when a mixture of Src antibodies to the N-terminus (2-17 mAb) and the SH3 domain (GD11) and 2500 μg lysate were used. Similar results were seen with the UACC-812 cell line. Thus, c-Src was detectable in the complexes precipitated from the MDA-MB-453 and UACC-812 cell lines, but in low amounts. A panel of c-Src-specific antibodies (directed to different regions of the c-Src molecule) and negative control antibodies was also used to determine specificity of the interaction. Figure 2, Panel C shows that co-immunoprecipitation of HER2 with c-Src was specific, as evidenced by the presence of HER2 in complexes with c-Src

and by the absence or significantly reduced levels of a similarly migrating protein in the negative antibody control precipitates.

Association of c-Src with HER2 in human breast tumor samples. Because HER family members and c-Src have been implicated in the formation of human breast tumors, we wished to determine whether c-Src/HER2 complexes existed in human breast carcinoma tissue or in normal breast tissue (as they did in established tumor cell lines), and if so, how frequently. A panel of thirteen tumor tissues, obtained from the University of Virginia and University of Michigan tumor banks, was analyzed by immunoprecipitation and Western blotting with antibodies to HER2 and c-Src, in a fashion similar to that used for the breast tumor cell lines. Figure 3 shows examples of this analysis. Tumors UVA156 and UVA263 exhibited c-Src/HER2 association, whereas MichN1 did not. In UVA263, reciprocal co-precipitation between c-Src and HER2 was observed. In addition, HER2/1 (UVA156 and UVA263) and HER2/3 (UVA156) complexes could be seen, indicating constitutive heterodimerization between HER family members in these tumors. Consistent with the data from the cell lines, this association was constitutive, suggesting the presence of an autocrine loop. Normal breast tissue contained detectable levels of c-Src but undetectable levels of HER2 and no association between HER2 and c-Src, HER1, or HER3. A protein of similar size as HER2 was sometimes seen to co-precipitate with the negative control antibody. The levels of this protein in control immunoprecipitates varied from experiment to experiment but were consistently less than that seen in the c-Src immunoprecipitates, and the protein exhibited slightly slower migration characteristics than did HER2 during gel electrophoresis.

Table II summarizes the results of such an analysis for all thirteen tumor samples and describes tumor type and grade, estrogen receptor (ER) status, lymph node involvement, relative

levels of c-Src and HER2 protein, and the presence or absence of a stable complex between c-Src and HER2 for each tumor. In all, stable association between c-Src and HER2 was seen in three out of thirteen samples, the same frequency as seen for the breast tumor cell lines. In contrast to the cell lines, however, c-Src/HER2 complexes were seen only in tissues that overexpressed both HER2 and c-Src. Complex formation between c-Src and HER2 has been demonstrated in mammary tumors in transgenic mice (33), but this is the first demonstration of an *in vivo* association in human breast carcinoma samples.

HRG-enhanced soft agar colony formation is ablated by the Src family inhibitor, PP1. In human breast tumor cell lines, HRG treatment has been demonstrated to increase tyrosine phosphorylation of many cellular proteins that are involved in mitogenic signaling pathways (52; Belsches-Jablonski, unpublished observations). It was therefore of interest to determine if HRG treatment affected the mitogenic or tumorigenic properties of these cell lines. To investigate this question, we chose a subset of the cell lines described in Table I, based on their varying expression of HER family members and c-Src. Three cell lines (MDA-MB-361, MDA-MB-453, and UACC-812) were selected because they co-overexpressed moderate to high levels of HER2 and HER3 and exhibited HER2/c-Src association. Of these, only MDA-MB-361 overexpressed c-Src. MCF-7, MDA-MB-468 and SK-BR3 cells were selected on the basis of their relatively low levels of HER2/HER3, moderate to high levels of c-Src, and lack of detectable HER2/c-Src complexes.

Figure 4 shows that only the cell lines that co-overexpressed HER2 and HER3 and contained c-Src/HER2 complexes (MDA-MB-361, MDA-MB-453, UACC-812) responded to HRG in a soft agar colony formation assay. HRG treatment potentiated anchorage-independent growth of these cell lines two to three-fold over that in 10% serum (Fig. 4, inset), while having no

significant effect on serum-induced colony formation of MCF7, MDA-MB-468, or SK-BR3 cell lines. Two of the latter three cell lines (MCF7 and MDA-MB-468) exhibited a robust colony forming activity that could not be augmented further by HRG, most likely since they are maximally activated by autocrine factors involving other signaling pathways (e.g., HER1/TGFα in the case of MDA-MB468 cells). SK-BR3 cells, in contrast, displayed poor growth in soft agar that was unaffected by HRG, suggesting that the signaling components to mediate HRG effects are either not present or are uncoupled in this cell line. As a whole, these data are consistent with the hypothesis that HRG-induced heterodimerization of HER2 and HER3 and the pre-existence of a c-Src/HER2 complex functioned together to promote a ligand-dependent enhancement of anchorage-independent growth, an indicator of tumorigenicity.

To test this hypothesis further, sister cultures of those depicted in the inset of Fig. 4 were treated with the Src family tyrosine kinase inhibitor, PP1, in the presence or absence of HRG and in the continual presence of 10% serum. Figure 4 shows that PP1 partially or completely inhibited HRG-enhanced soft agar growth in MDA-MB-361, MDA-MB-453, and UACC-812 cells, as well as variably reducing growth in serum plus HRG in MCF7, MDA-MB-468, and SK-BR3 cells. Furthermore, PP1 had a striking inhibitory effect on the ability of all cell lines to form colonies in serum alone, suggesting that PP1 is capable of affecting other growth pathways in addition to those that are HRG-induced. The results of this experiment could also be interpreted to mean that HRG "rescues" PP1-induced inhibition, again to variable extents in the various cell lines.

HRG-potentiated anchorage-dependent growth is partially dependent on Src family kinases. Because HRG treatment led to an increase in anchorage-independent growth of the three cell lines that co-overexpressed HER2 and HER3 and contained constitutive c-Src/HER2 complexes, we wished to determine whether HRG also affected anchorage-dependent growth of

these and other cell lines. An MTT assay was used to follow cell number as a function of incubation time in the various treatment conditions. No effect of HRG on the cell number of any of the cell lines was observed in 10% serum, even when assessed up to two weeks after plating (data not shown). However, Figure 5 shows that HRG treatment resulted in small, but significant increases in numbers of MDA-MB-361, UACC-812, and MCF7 cells, when they were maintained in reduced serum (0.5%) over a five day period (Panels A, B, C; compare "day 5" to "day 5+H"). Interestingly, the effect of HRG on cell number in this anchorage-dependent assay did not correlate with the presence of a c-Src/HER2 complex nor with levels of the various HER family members.

PP1 treatment prevented growth or reduced the cell number of all cell lines maintained in low serum alone (Fig. 5; compare "day 5 + P" to "day 5" in each panel), but the effect of PP1 on cells treated with HRG varied from cell line to cell line. HRG-treated MDA-MB-361 and MCF7 lines were only slightly reduced in cell number by PP1 treatment (Fig.5, Panels A and C; compare "day 5 + H + P" to "day 5 + H"), whereas UACC-812 was reduced to levels equal to or below that of cells maintained in 0.5% serum alone (Panel B). These results suggest that c-Src and/or related family members cooperate with HRG to promote anchorage-dependent growth and/or survival of the breast cancer cell lines tested, but the extent of this cooperation varies from cell line to cell line. Results similar to those for MCF7 cells were found for MDA-MB-468 and SK-BR3 cells. MDA-MB-453 cells did not respond to HRG in this assay (data not shown).

PP1 has previously been shown to inhibit Src family members, Fyn and Lck (42). To determine whether PP1 also inhibited c-Src kinase activity, immune complexes of c-Src that had been prepared from MDA-MB-361 were preincubated for 15 min with varying concentrations of

PP1 (ranging from 0.1 μM to 10 μM) before the auto- and trans-phosphorylating activities were assessed in an *in vitro* immune complex kinase assay, using heat-denatured enolase as an exogenous substrate and/or [γ-^{32}P]-ATP. Both the auto- and trans-phosphorylating activities of c-Src were reduced by PP1 in a dose- and time-dependent manner (data not shown). The IC_{50} was 0.3 - 1.0 μM PP1 in a 10 min incubation. These results verified that PP1 is an efficient inhibitor of c-Src kinase activity.

To further assess the role of c-Src and its family members on the growth and viability of breast cancer cells, we attempted to generate stable transfectants of MDA-MB-361 cells that expressed a dominant negative, kinase-deficient form of chicken c-Src (V430A) (53) that had been subcloned into the pcDNA3 mammalian expression vector (Invitrogen; 54). Transfected cells were selected for neomycin resistance, and early, antibiotic-resistant populations of cells were shown to express high levels of the variant form of c-Src by Western blotting with the chicken c-Src-specific mAb, EC10. However, no long-term stable clones could be obtained from this population, even after repeated efforts (data not shown). These data suggest a striking dependence on c-Src kinase activity for long-term survival and growth of these cells, even in full serum conditions. Not all human breast cancer cell lines behaved as did MDA-MB-361 cells, in that stable clones expressing kinase-defective c-Src could be obtained from them. Examples of such cell lines include MDA-MB-468 and MDA-MB-231. Similar to MDA-MB-361 cells, however, their tumorigenic properties were impaired (54).

Heregulin reverses the apoptotic effect of PP1 in reduced serum. Because the effect of HRG on anchorage-dependent growth in reduced serum was so modest and PP1 had such a deleterious effect on the growth and viability of the majority of cell lines tested (which was partially or completely reversed by HRG), we examined several of the cell lines (MDA-MB-361, UACC-812,

and MCF7) for possible evidence of an apoptotic effect of PP1 and an anti-apoptotic effect of HRG. Cells were cultured for three days in the presence or absence of HRG and/or PP1 and analyzed for evidence of apoptosis, using DAPI-staining of DNA to detect abnormally shaped nuclei and/or condensed chromatin (46). Figure 6, Panel A shows that PP1 treatment of MDA-MB-361 cells induced apoptosis in 10-15% of the cells (vs. ~2% in 0.5% serum alone) and that PP1-induced apoptosis was almost completely reversed by HRG. UV-induced apoptosis was used as a positive control (>85%). Similar results were obtained when UACC-812 and MCF7 cells were examined, except that the extents of reversal differed slightly (Fig. 6, Panels B and C). In all cell lines, the anti-apoptotic effects of HRG were found to be insensitive to Wortmannin, suggesting a PI-3 kinase-independent mechanism of action by HRG. Results of the DAPI analysis were verified with the TUNEL assay for apoptosis (data not shown).

DISCUSSION

Both HER family members and c-Src are overexpressed at high frequency in human breast cancers (25, 30, 32, 37, 50, this report) and have been implicated in the genesis and progression of the disease. Amplification/overexpression of HER2, especially, has been associated with a poor patient prognosis (25, 30). Synergistic interactions between c-Src and HER1 in tumor formation have recently been reported (36, 37), and c-Src has been shown to be required for EGF-induced mitogenesis (53, 55), suggesting that similar interactions may exist between c-Src and other HER family members. Here we describe the results of experiments designed to investigate this issue in the context of human breast tumor cell lines and tumor tissue. In these studies, tumor cell lines were treated with ligands that activate HER3/4 and induce homo- and hetero-dimerization with HER2 as well as with an inhibitor of Src family kinases, PP1, to

determine if c-Src or related kinases contribute to HRG-initated events. We found that HRG-enhanced colony formation in soft agar and adherence-dependent proliferation are inhibited by PP1, suggesting that Src family members participate in HRG-mediated growth processes in human tumor cells. However, we also found that the extent of the effects of PP1 (and thus presumably the effects of Src family kinases) are cell-line dependent, i.e., partial inhibition in some cell lines and complete inhibition in others (Figs. 4 and 5). Heregulin was also shown to reverse the growth inhibitory or apoptotic effects of PP1 (Figs. 4-6). Together, these results indicate that Src family kinases participate in HRG-regulated cellular processes and vice-versa, but the extent to which this occurs is likely to be determined by the unique signaling circuitry of each tumor cell line. These results are also suggestive of Src-independent as well as Src-dependent mechanisms for HRG action.

One mechanism by which HRG may be dependent on Src family members is through physical association of HER2 with c-Src. In such a scenario, c-Src might be thought to function by facilitating formation of heterocomplexes between HER2 and other HER family members or by phosphorylating HER2 or one of its receptor partners, as has been shown for HER1 (36, 40). These possibilities warrant further testing in a non-tumorigenic cell system, where the constituents of the complex can be regulated by overexpression and tumor-specific modifications to the receptor are eliminated.

In 23% of cell lines and tumor tissues tested (3/13 in each case), a stable and specific association between c-Src and HER2 was observed. In each of the cell lines exhibiting this association, HER3 is present and HER4 is undetectable (Fig. 1, Table I). These data argue that the functional complex mediating growth potentiation effects in soft agar assays is comprised of c-Src, HER2, and HER3 (12-15). The ability to detect c-Src/HER2 complexes correlated with the

ability of HRG to promote colony formation in soft agar, suggesting that the complex may participate in anchorage-independent growth. Involvement of c-Src in such a process is consistent with its documented roles in regulating focal adhesion formation (cell-substratum interactions) and inter-cellular interactions via cadherins (reviewed in 56, 57). Indeed, we have observed complete inhibition of p190RhoGAP, p130CAS, and cortactin tyrosine phosphorylation in five different human breast tumor cell lines treated with PP1 (D. Tice and S. Parsons, unpublished observations). These proteins are all demonstrated substrates of c-Src and involved in signaling pathways that regulate the actin cytoskeleton (reviewed in 58) .

However, the ability to detect c-Src/HER2 complexes in the various breast tumor cell lines did not correlate with the anchorage-dependent growth-promoting or the anti-apoptotic effects of HRG (Table I, Figs. 5 and 6). Furthermore, in some cell lines and tissues where the complex was found, the stoichiometry of the interaction was low, raising questions about its physiological significance. These considerations suggest that Src family members may communicate with HER2 via other mechanisms. Alternative mechanisms include the following: The first presupposes that in some cell types HER2 and c-Src do not physically interact but are members of the same pathway and that HRG treatment indirectly induces changes in c-Src or its related family members which results in transmission of the growth or survival signal to downstream components. Given the result that HRG can reverse the apoptotic effect of PP1 (Fig. 6), HRG could be viewed as having the capability to override the inhibitory action of PP1 on c-Src or other family members. In this situation, HRG could function either upstream or downstream of c-Src, the latter through a feedback loop. In either case, we have found that the action of PP1 on Src family members is reversible both *in vivo* and *in vitro* (Belsches-Jablonski, Cox, and S. Parsons, unpublished), indicating that such a competitive action of HRG is possible.

Another potential mechanism of Src/HER2 interaction suggests that the two kinases are members of different pathways, which converge downstream of the points of action of c-Src and HER2. Since c-Src and its related family members are known to be involved in multiple growth factor-regulated responses (EGF, PDGF, G protein-coupled [53, 58-62]), such pathways could converge on HRG-stimulated pathways downstream of the action of Src. In favor of this possibility is our inability to detect c-Src activation in response to HRG treatment (data not shown), a finding that is consistent with the notion that c-Src does not lie directly downstream of HRG receptors.

Alternatively, HRG could be activating Src family members other than c-Src. Fyn and Yes have been found to be activated in certain types of human tumors, such as colon (63, 64) and lung (65, 66). However, these family members appear to be less frequently activated or overexpressed in human breast cancer cells or tissues than is c-Src (37; reviewed in 67), suggesting that c-Src is an appropriate focus of our attention for these studies.

Despite the structural similarities between HER1 and HER2 and their involvement in the genesis of breast cancer and other human tumors, there are striking differences between c-Src/HER1 and c-Src/HER2 complexes. In both a murine fibroblast model system and in a subset of human breast tumor cell lines, physical association of c-Src with HER1 is EGF-dependent, and was found to occur only in the presence of overexpression of both c-Src and HER1 (36, 37). In contrast, in human tumor cell lines, the formation of HER2/c-Src complexes is constitutive, not dependent upon overexpression of c-Src, and insensitive to HRG treatment. Given the many ligands that have been shown to bind to HER family members, it is reasonable to speculate that autocrine production of ligands could cause constitutive activation of the receptors and thus association with c-Src. In support of this is our finding that p-Tyr-containing proteins in the 170-

185 kDa range are seen in p-Tyr immunoblots of unstimulated lysates from each cell line which contains the c-Src/HER2 complex (data not shown). Treatment of these cell lines with EGF or HRG did not increase the overall amount of p-Tyr signal, consistent with the notion that the endogenous receptors were already activated.

The correlation between the ability to detect a c-Src/HER2 complex in certain cell lines and to observe a biological effect on HRG-induced growth in soft agar suggests that this complex may be important in promoting malignant transformation. The functional linkage between Src family kinases and HRG in both the anchorage-independent and -dependent growth assays provides additional support for the hypothesis that c-Src and HER2 co-operate at many levels to increase tumorigenicity, as we and others have demonstrated for c-Src and HER1 (36, 37, 40, 41, 58, 67). However, from available evidence we believe that the mechanism of co-operativity between c-Src and HER2 will be revealed to be different from that of c-Src and HER1. For example, we have not observed a common downstream signaling molecule that is activated in the various breast tumor cell lines by HRG, while Shc and MAP kinase have been routinely found to be activated in many of those same cell lines by EGF (37; Belsches-Jablonski and S. Parsons, unpublished). Current efforts are directed toward understanding the nature of the physical interaction between c-Src and HER2 (direct or indirect) and the signaling pathways that convey their overlapping and unique directives to the nucleus.

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FIGURE LEGENDS

Figure 1. Overexpression of HER family members and c-Src in human breast carcinoma cell lines. Lysates from indicated cell lines were prepared in RIPA buffer, and 100 μg protein was loaded per well in a 7% polyacrylamide gel. Western blots were probed with antibodies to c-Src (2-17); HER1 (3A and 4A); HER2 (Santa Cruz SC-284); HER3 (Santa Cruz SC-285); and HER4 (Santa Cruz SC-283/UBI #06-572). Primary antibodies were visualized by ^{125}I-protein A, except for the HER4 antibodies, which were detected with ^{125}I-goat anti-rabbit IgG. Portions of this figure are reproduced from ref. 37, Copyright 1998, Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

Figure 2. Physical association between c-Src and HER2 in human breast carcinoma cell lines. Panel A: MDA-MB-361, MDA-MB-453, MCF7 and MDA-MB-468 cells were serum-starved for 17 hr. and either stimulated with 200 ng/ml HRG-α (+) or incubated in fresh serum-free media (-) for 5 min. Cells were then lysed, and 500 μg lysate protein from each cell line and treatment protocol was incubated with either anti-Src (S) (2-17 mAb), anti-HER2 (H2) or mouse IgG. Precipitated proteins were separated by SDS-PAGE and transferred to a nylon membrane. The upper half of the membrane was probed with anti-HER2 primary antibody, and the lower half was probed with anti-Src (2-17) primary antibody. Both primary antibodies were visualized by ^{125}I-protein A. **Panel B.** MDA-MB-361 and MDA-MB-453 cells were treated as in (**A**), except that 2.5 mg lysate protein and a mixture of 2-17 and GD11 c-Src-specific mAbs were used for the immunoprecipitation. The nylon membrane was probed with 2-17 mAb. Primary antibody was visualized by ^{125}I-protein A. **Panel C.** Lysates from unstimulated MDA-MB-361 cells were prepared as in (**A**), and 1.0 mg lysate protein was immunoprecipitated with each of four different antibodies to c-Src (see Materials and Methods), as well as mouse anti-rabbit (MAR) or mouse IgG as negative antibody controls. Immunoblots were prepared as in (**A**).

Figure 3. Presence of endogenous c-Src/HER2 complexes in human breast tumor tissue. Tumor lysates were homogenized, lysed in RIPA detergent buffer, and clarified by centrifugation. Cellular proteins were immunoprecipitated with anti-Src (S), anti-HER1 (H1), anti-HER2 (H2), or anti-HER3 (H3) antibodies from 250 μg lysate protein. Precipitated proteins were separated on 7% polyacrylamide gels and transferred to nylon membranes. The upper half was probed with

anti-HER2, and the lower half with 2-17 mAb. Both primary antibodies were visualized by ^{125}I-protein A. (-) indicates mouse anti-rabbit IgG as a negative control for immunoprecipitation.

Figure 4. Effects of HRG and the Src kinase family inhibitor, PP1, on anchorage-independent growth of human breast tumor cells. MDA-MB-361, MDA-MB-453, UACC-812, MCF7, MDA-MB-468 and SK-BR3 cells were plated in soft agar as described in Materials and Methods and incubated at 37^{0}C in a humidified, 5% CO_2 atmosphere for two weeks in DMEM containing the following: 10% serum + DMSO vehicle control; 10% serum + 40 ng/ml HRG-α + DMSO vehicle control; 10% serum + 40 ng/ml HRG-α + 10 μM PP1; or 10% serum + 10 μM PP1. Media with additives was replenished every 4 days. Colonies were stained, counted, and expressed as the mean colony number ± SEM of three independent experiments, each performed in triplicate. Inset depicts fold difference of HRG treatment over non-stimulated controls. * = significantly different than 10% serum alone (p-values ≤ 0.05).

Figure 5. Effects of HRG and PP1 on anchorage-dependent, reduced-serum growth and survival of human breast cancer cells. MDA-MB-361 (**Panel A**), UACC-812 (**Panel B**), and MCF7 (**Panel C**) cells were plated in DMEM with the following additives: 0.5% serum + DMSO vehicle control; 0.5% serum + 40 ng/ml HRG-α or -β + DMSO vehicle control; 0.5% serum + 40 ng/ml HRG + 10 μM PP1; or 0.5% serum + 10 μM PP1, as described in Materials and Methods. The cultures were incubated for 5 days, with replenishment of media containing the appropriate additives at day 2.5. Cell number at Days 0 and 5 was determined by the MTT assay and is expressed as mean fold-change ± SEM relative to Day 0. Day 0 density was determined 12 hr after plating. Results are pooled from 4 independent experiments, each performed in triplicate. #

= significantly different than day 5, 0.5% serum alone. ψ = significantly different than day 5, 0.5% serum + HRG. p-values ≤ 0.05.

Figure 6. Anti-apoptotic effects of HRG and Src family kinases in human breast tumor cell lines. Cells were plated onto glass coverslips (5 X 10^4 cells/coverslip) and grown in one of the following, DMEM plus: 0.5% serum alone + DMSO vehicle control; 0.5% serum + 40 ng/ml HRG-α + DMSO vehicle control; 0.5% serum + 10 μM PP1; or 0.5% serum + 40 ng/ml HRG-α + 10 μM PP1, and incubated for 3 days. Cells were then fixed in 4% paraformaldehyde and permeabilized, and nuclei were stained for DNA with DAPI. At least 400 cells per treatment were counted for apoptotic nuclei in two separate experiments. A 2-min UV-B treatment and 48 hr recovery in serum-free media was used as a positive control for apoptosis. **Panel A**: MDA-MB-361; **Panel B**: UACC-812; **Panel C**: MCF7. * = significant difference between 0.5% serum or 0.5% serum + HRG + PP1 and 0.5% serum + PP1 (p-values ≤ 0.05).

Table I†

Human Breast Carcinoma Cell Lines

Relative levels of HER family members and c-Src

						Association of c-Src with:		
cell line	HER1	HER2	HER3	HER4	c-Src	ER	HER1	HER2
MDA-MB175	*	2.2	6.1	*	9.9	+	-	-
UACC-893	*	72.8	6.7	*	*	-	ND	ND
UACC-812	*	50.8	6.1	*	*	-	-	+
SK-BR-3	12.6	2.5	*	1.0	19.4	-	+	-
MDA-MB361	*	12.8	10.9	*	37.4	+	-	+
MDA-MB453	*	1.5	6.1	*	*	-	-	+
MDA-MB468	39.5	*	*	*	4.9	-	+	-
ZR75-B	*	*	1.1	4.4	6.2	+	-	-
BT-474	*	61.8	26.4	6.4	*	+	-	-
BT-549	6.9	*	*	*	13.4	-	+	-
MDA-MB231	7.6	1.1	1.0	*	2.9	-	+	-
BT-20	3.4	1.9	5	*	13.5	-	+	-
MCF-7	1	1	2.3	1.5	6.4	+	-	-
Hs578Bst[1]	1	*	*	*	1	+	-	-

(*) = below limits of detection ND in table indicates no data.

†Autoradiographs from Figure 1 were analyzed by densitometry and compared to Hs578Bst[1], an immortalized normal human mammary epithelial cell line. The lowest detectable signal on each blot was set as 1.0, and fold difference of the cell lines was determined. An * indicates a signal was below the limits of detection by Western blotting. Also included in the table are estrogen receptor (ER) status of the cell lines, as determined by Western blotting (37), and physical association between c-Src and either HER1 or HER2, as determined by immunoprecipitation with monoclonal antibodies to c-Src. Portions of this table are re-printed from ref. 37, Copyright 1998, Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc..

Table II[†]

Summary of Human Breast Tumors Tested for Physical Association of HER2 and c-Src

Tumor	Tumor Type	ER	Lymph Node	Src/HER2 Association	RELATIVE LEVELS c-Src	HER2
UVA103	IVDC[1], grade 3/3	-	-	-	ND[6]	ND
UVA156	IDC[2], grade 2/3	-	+	+	+++	+++
UVA226	IDA[3], grade 2/3	+	ND	+	++	+++
UVA263	IDC, grade 3/3	ND	+	+	+++	+++++
UVA387	ILC[4]	+	ND	-	+	*[7]
UVA399	IDC, grade 3/3	-	ND	-	+	+++
UVA454	IDC, grade 3/3	-	+	-	++	++
UVA616	IVDC, grade 3/3	-	+	-	ND	ND
MICHN1	medullary, grade 3/3		ND	-	+	++
MICHP1	#[5]	ND	ND	-	*	++
MICHP2	#	ND	ND	-	*	+
MICHP3	#	ND	ND	-	*	+
MICHP4	#	ND	ND	-	*	+

[†]Human breast tumor samples obtained from the UVA Tissue Procurement Facility and the University of Michigan Tumor Bank were analyzed by immunoprecipitation and Western blotting for physical association between HER2 and c-Src. Tumor type and grade, ER and lymph node status, and relative levels of HER2 and c-Src protein are listed for each tumor.

[1]IVDC = invasive ductal carcinoma

[2]IDC = infiltrating ductal carcinoma

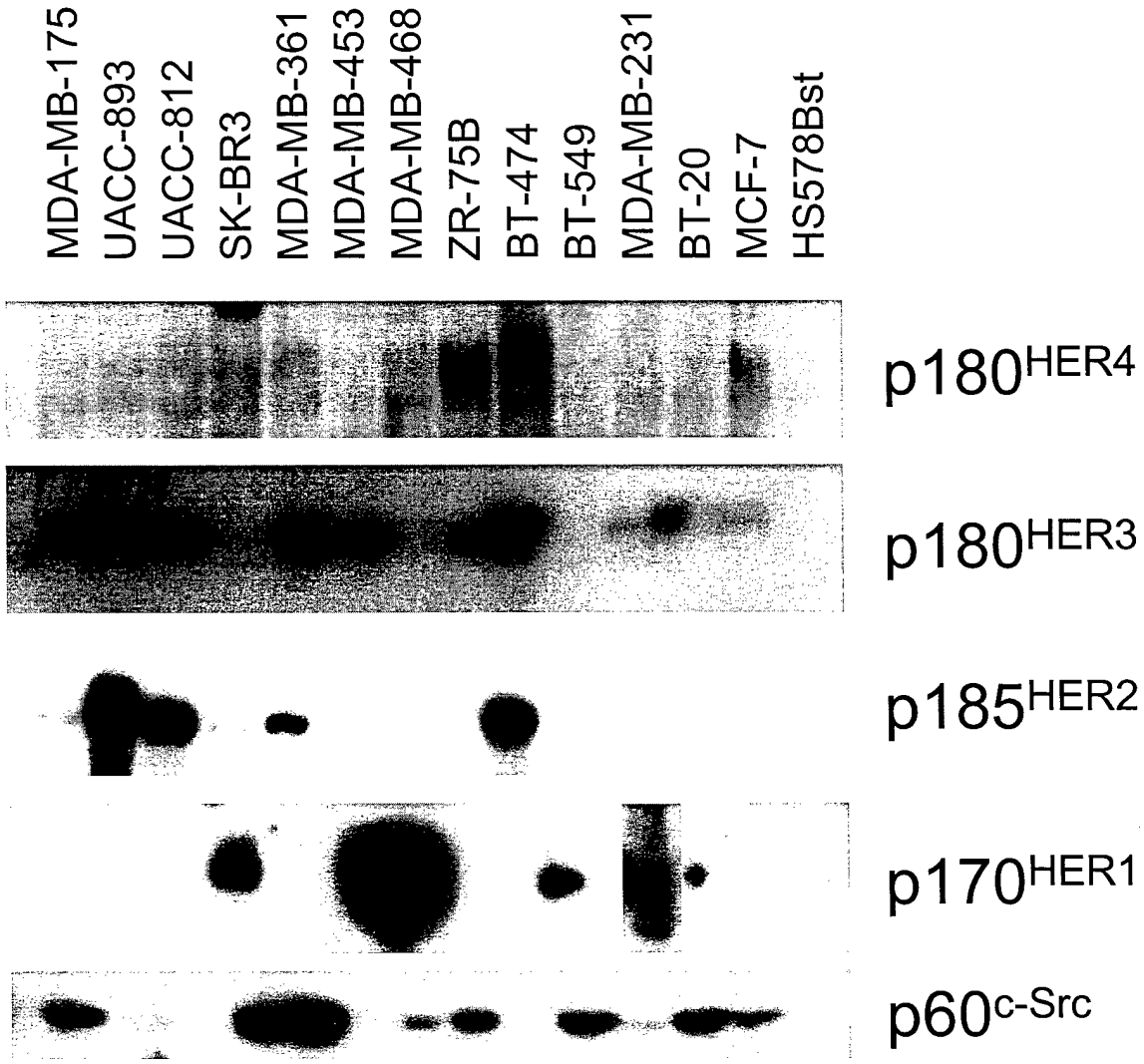
[3]IDA = infiltrating ductal adenocarcinoma

[4]ILC = infiltrating lobular carcinoma

[5]# = data currently not available

[6]ND = no data

[7]* = below limits of detection


MDA-MB-175
UACC-893
UACC-812
SK-BR3
MDA-MB-361
MDA-MB-453
MDA-MB-468
ZR-75B
BT-474
BT-549
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BT-20
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HS578Bst
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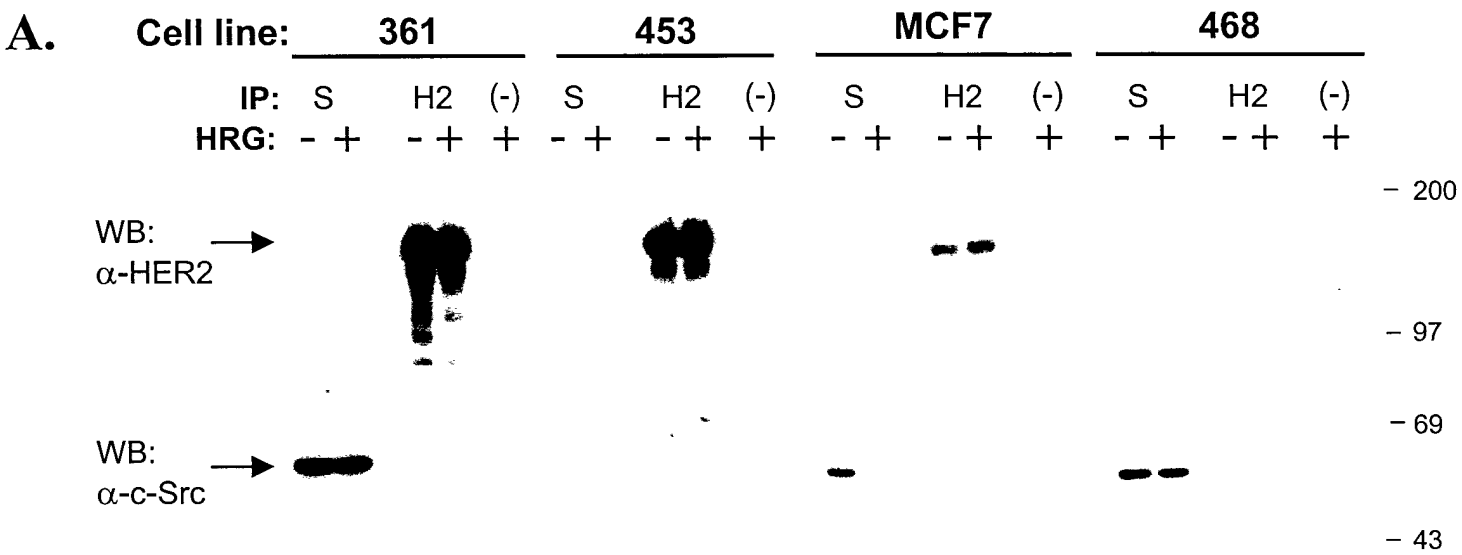
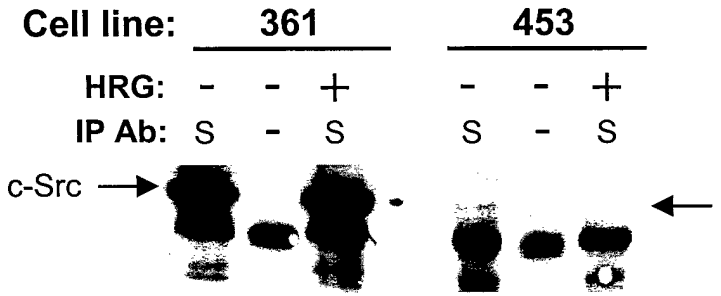
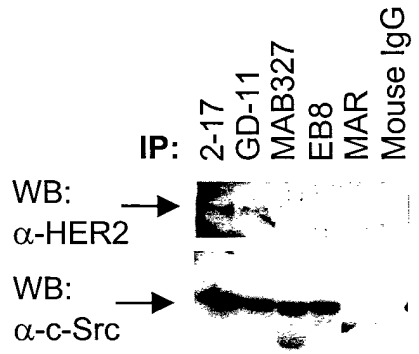
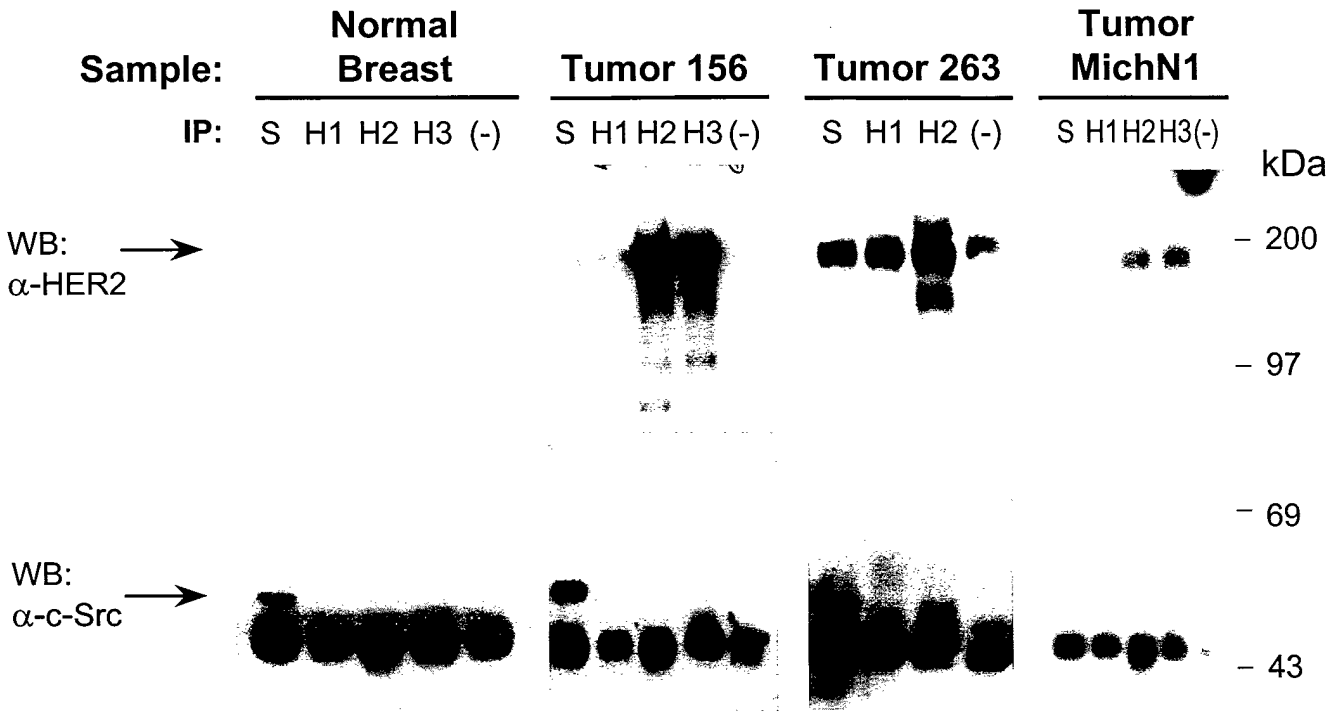
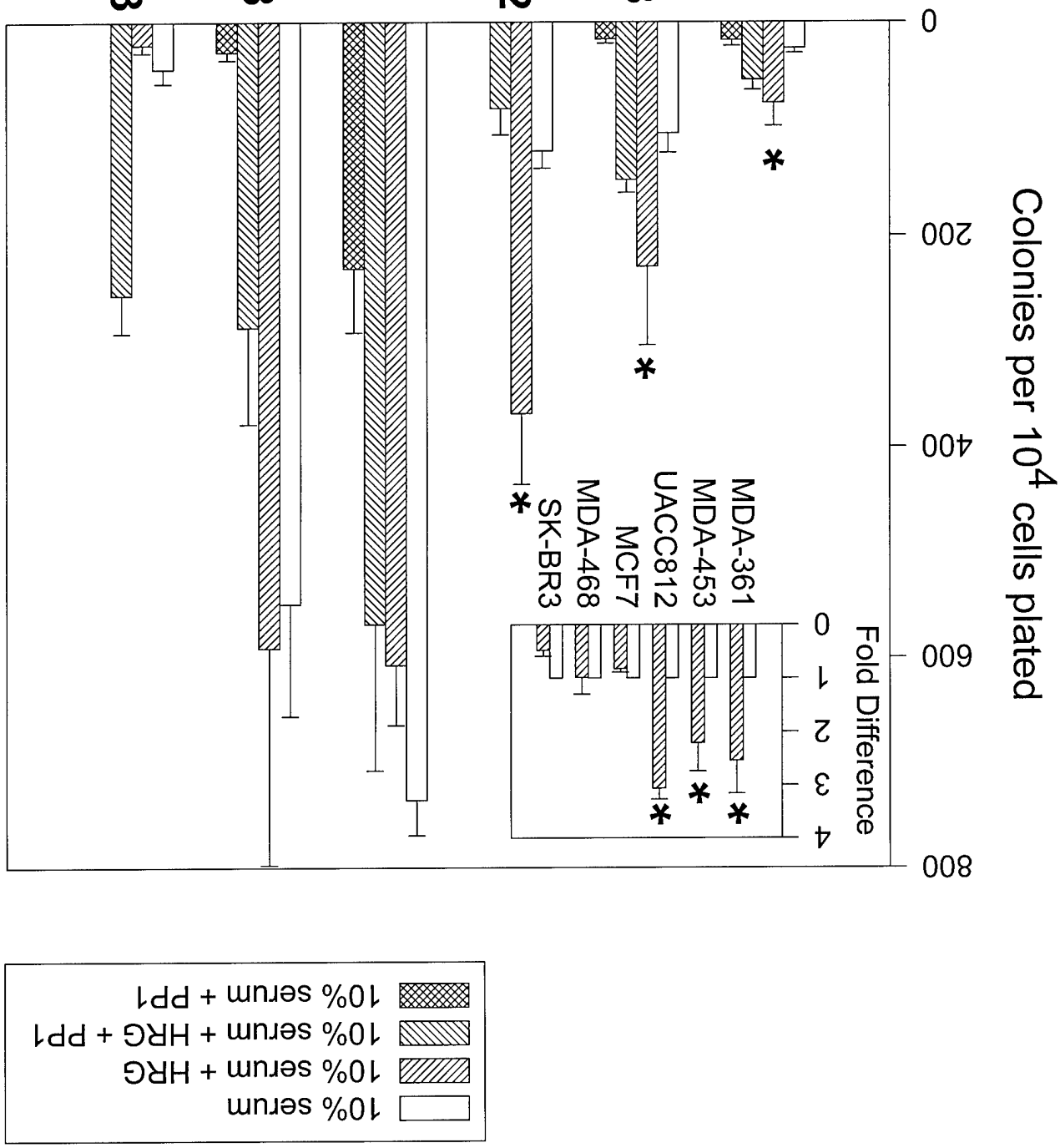
p180HER3
p185HER2
p170HER1
p60c-Src


A.
Cell line: 361 453 MCF7 468
IP: S H2 (-) S H2 (-) S H2 (-) S H2 (-)
HRG: - + - + + - + - + + - + - + + - + - + +
WB: α-HER2
WB: α-c-Src
200
97
69
43


B.
Cell line: 361 453
HRG: - - + - - +
IP Ab: S - S S - S
c-Src


C.
IP: 2-17 GD-11 MAB327 EB8 MAR Mouse IgG
WB: α-HER2
WB: α-c-Src


Sample:
Normal Breast
Tumor 156
Tumor 263
Tumor MichN1
IP:
S H1 H2 H3 (-)
S H1 H2 H3 (-)
S H1 H2 (-)
S H1 H2 H3 (-)
kDa
WB:
α-HER2
WB:
α-c-Src
200
97
69
43


Colonies per 10^4 cells plated
0
200
400
600
800
MDA-361
MDA-453
UACC812
MCF7
MDA-468
SK-BR3
Fold Difference
0
1
2
3
4
10% serum
10% serum + HRG
10% serum + HRG + PP1
10% serum + PP1

A.
MDA-MB-361
Cell Number
(fold change over Day 0)
0
1
2
3
4
day 0
day 5
day 5+H
d.5+H+P
day 5+P
#
#
#
B.
UACC-812
Cell Number
(fold change over Day 0)
0
1
2
3
4
day 0
day 5
day 5+H
d.5+H+P
day 5+P
#
Ψ
#
#
C.
MCF7
Cell Number
(fold change over Day 0)
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3
4
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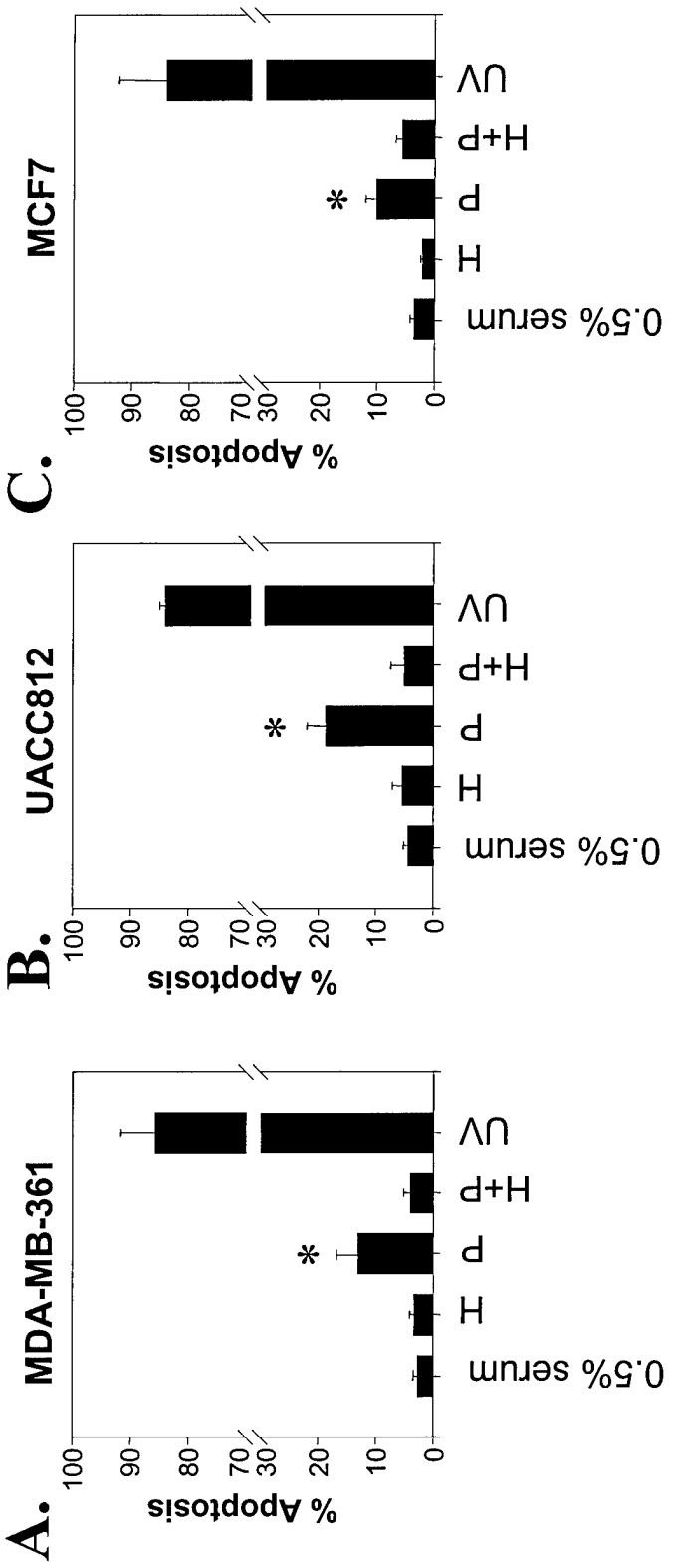
day 5
day 5+H
d.5+H+P
day 5+P
#
#


A.
MDA-MB-361
B.
UACC812
C.
MCF7
% Apoptosis
0
10
20
30
70
80
90
100
0.5% serum
H
P
H+P
UV
*

FINAL REPORTS

- **Publications:** The manuscript entitled "Src Family Kinases and Heregulin Cooperate to Promote Human Breast Cancer Cell Growth and Survival," was submitted to Cancer Research.

- **Meetings Attended:**

1. "Basic and Clinical Aspects of Breast Cancer", an AACR meeting at Keystone, CO, March, 1997. Poster.
2. The DOD breast cancer meeting, "An Era of Hope", October, 1997. Poster.
3. Oncogene meeting, June 1997. Poster.
4. Pathways to Cancer, Cold Spring Harbor, March 1998. Poster.
5. "Specificity in Signaling", Keystone Symposium, Keystone, CO, April 1999. Poster.
6. Oncogene meeting, June 1999. Oral presentation and Poster.

- **Meeting Abstracts:**

1. "Basic and Clinical Aspects of Breast Cancer", Keystone Abstract, 1997

FUNCTIONAL AND STRUCTURAL INTERACTIONS BETWEEN C-SRC AND HER2: INVOLVEMENT IN HUMAN BREAST TUMOR FORMATION. Allison P.Belsches, J.S.Biscardi, S.J.Parsons. Dept. of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA.

Previous studies by several laboratories have shown that functional interaction between members of the human EGF receptor (HER) receptor tyrosine kinase family, and c-Src, a non-receptor tyrosine kinase, may be involved in breast tumor formation. Our laboratory has demonstrated that overexpression of two tyrosine kinases, HER1 and c-Src, in a murine fibroblast cell line, C3H10T1/2, results in an EGF-dependent synergistic increase in tumor formation in nude mice, as compared to overexpression of either protein alone. This increase in tumorigenicity correlates with the formation of an *in vivo* complex between HER1 and c-Src, the appearance of two novel phosphorylation sites on HER1, and enhanced phosphorylation of receptor substrates, suggesting that association of the HER1 receptor with c-Src causes a hyperactivation of receptor kinase activity. Hyperactivation of the HER1 receptor might then lead to enhanced intracellular signaling, resulting in augmented growth. The HER family of growth factor receptors, including HER1 and HER2, exhibit a high percentage of structural homology. HER2 is overexpressed in approximately 30% of human breast cancers, and this overexpression is correlated with poor patient prognoses. In addition, elevated levels and/or activity of c-Src have been demonstrated in human breast cancers. Because of their structural similarity and elevated expression in human breast cancers, we propose that HER1 and HER2 interact with c-Src through similar mechanisms to augment cellular growth and tumor progression. Existing data from other laboratories support this hypothesis. Wildtype HER2 has been shown to be weakly tumorigenic when overexpressed in NIH-3T3 cells (Science 237:178-182), and stable HER2/c-Src complexes have been detected in human and rodent mammary tumor cell lines and tissues. To examine the potential interactions between HER2 and c-Src, our approach has been two-fold. First, we have studied human breast tumor tissue, as well as a panel of human breast carcinoma cell lines, for expression of HER family members and c-Src by Western blotting. In almost all samples, either HER1 or HER2 is overexpressed, but not both simultaneously. These data are consistent with the hypothesis that different HER family members are expressed at different times during tumor progression, with HER2 being expressed primarily in earlier stage tumors, and HER1 being expressed in later, more invasive tumors. Lysates from tumors and cell lines have also been tested for heterocomplexes containing c-Src and HER2. Of thirteen tumor tissues examined, three exhibited heterocomplex formation, and in three of nine human breast tumor cell lines which overexpress both HER2 and c-Src, immunoprecipitation assays indicate an *in vivo* complex formed between c-Src and HER2,

independent of EGF stimulation. The finding of c-Src/HER2 complexes in known tumor and carcinoma cell lines suggests structural and functional interactions between HER2 and c-Src. The second approach has been to test the hypothesis that HER2 and c-Src interact synergistically to potentiate tumor formation and growth, by generating model cell lines in a known genetic background (using C3H10T1/2 murine fibroblasts as a parental line), which overexpress wildtype HER2 and c-Src, either separately or in combination, and testing them for growth properties, heterocomplex formation and downstream signaling events. Preliminary results indicate an immune complex of HER2 and c-Src in a cell line which overexpresses both tyrosine kinases. No complex between HER2 and c-Src is seen in a cell line expressing c-Src alone. Cell lines generated will be tested for tumorigenicity in assays of [^{3}H]-thymidine incorporation, growth in soft agar, and tumor formation in nude mice. Performed both in model systems and in breast cancer cell lines, these investigations may identify potential mechanisms of action between HER family members and c-Src, as well as target molecules active in breast cancer formation and progression.

2. DOD Abstract, 1997

FUNCTIONAL AND STRUCTURAL INTERACTIONS BETWEEN C-SRC AND HER2: INVOLVEMENT IN HUMAN BREAST TUMOR FORMATION. Allison P. Belsches, J.S. Biscardi, D. Peavy, S.J. Parsons. Dept. of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA 22908

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c-Src alone. Cell lines generated will be tested for tumorigenicity in assays of [^{3}H]-thymidine incorporation, growth in soft agar, and tumor formation in nude mice. Performed both in model systems and in breast cancer cell lines, these investigations may identify potential mechanisms of action between HER family members and c-Src, as well as target molecules active in breast cancer formation and progression.

FUNCTIONAL AND STRUCTURAL INTERACTIONS BETWEEN C-SRC AND HER2: INVOLVEMENT IN HUMAN BREAST TUMOR FORMATION. Allison P. Belsches, J.S. Biscardi, D. Peavy, S.J. Parsons. Dept. of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA 22908

Breast cancer is estimated to affect one in nine women in their lifetime. There is understandably a great effort to find the cause of breast cancer. In studies of breast tumor cells, certain proteins have been shown to be produced in greater amounts than in normal breast tissue, particularly in more advanced stage tumors. Because these proteins are found in greater abundance in cancerous breast tissue rather than in normal tissue, it is believed that they may be involved in the formation and progression of breast cancer. Previous work in our laboratory has shown that interactions between two of these proteins, c-Src and HER1, cause increased malignant growth in mice. This project has examined c-Src and a protein very similar to HER1, known as HER2, in a panel of breast tumor cells and actual patient breast tumor samples, and tested for physical interactions between the proteins. We have demonstrated a physical association between c-Src and HER2, in at least three breast tumor cell lines and in three of thirteen patient breast tumor samples. A model cell system is currently being constructed to test if overproduction of both of these proteins can directly cause the cells to grow uncontrollably. Eventually, we will design small molecules to interrupt the physical association of these proteins in an attempt to prevent the formation of breast tumors. This potential therapy may be important to breast cancer patients whose tumors overproduce c-Src and HER2, to prevent additional tumors from forming.

3. Oncogene Meeting Abstract, 1997

FUNCTIONAL AND STRUCTURAL INTERACTIONS BETWEEN C-SRC AND HER2/*neu*: INVOLVEMENT IN HUMAN BREAST TUMOR FORMATION. Allison P. Belsches, J.S.Biscardi, D.Peavy, S.J.Parsons. Dept. of Microbiology and Cancer Center, University of Virginia Health Sciences Center, Charlottesville, VA.

Previous studies have shown that functional interaction between members of the human EGF receptor (HER) receptor tyrosine kinase family, and c-Src, a non-receptor tyrosine kinase, may be involved in breast tumor formation. Our laboratory has demonstrated that overexpression of HER1 and c-Src, in a murine fibroblast cell line, C3H10T1/2, results in an EGF-dependent synergistic increase in tumorigenicity, as compared to overexpression of either kinase alone. This increase in tumorigenicity correlates with formation of an *in vivo* HER1/c-Src complex, the appearance of two novel phosphorylation sites on HER1, and enhanced phosphorylation of receptor substrates, suggesting that association of the HER1 receptor with c-Src causes a hyperactivation of receptor kinase activity, leading to enhanced intracellular signaling, and augmented growth. The HER family of receptors, including HER1 and HER2, exhibits extensive structural homology. HER2 is overexpressed in 20-30% of human breast cancers, and is correlated with poor patient prognosis. Also, elevated levels and/or activity of c-Src have been demonstrated in human breast cancers. Given this, we propose that HER1 and HER2 interact with c-Src through similar mechanisms to augment cellular growth and tumor progression Wildtype HER2 has been shown to be weakly tumorigenic when overexpressed in NIH-3T3 cells, and stable HER2/c-Src complexes have been detected in human and rodent mammary tumor cell lines and tissues. To examine potential interactions between HER2 and c-Src, our approach has been two-fold. First, we have studied human breast tumor tissue, and a panel of human breast carcinoma cell lines, for expression of HER family members and c-Src by Western blotting. In almost all samples, either HER1 or HER2 is overexpressed, but not simultaneously. Lysates of tumor samples and cell lines were also tested for HER2/c-Src heterocomplexes. In 3 of 13 tumor tissues, and in 3 of 9 human breast tumor cell lines, immunoprecipitation assays indicated the presence of *in vivo* HER2/c-Src complexes, which form independently of EGF or heregulin stimulation. The finding of HER2/c-Src complexes in human breast tumors and carcinoma cell lines suggests structural and functional interactions between HER2 and c-Src. A second approach has been to test if HER2 and c-Src interact synergistically to potentiate tumor formation

and growth by generating cell lines (using C3H10T1/2 murine fibroblasts as a parental line) which overexpress *wt* HER2 and/or c-Src and testing them for growth properties, heterocomplex formation and downstream signaling. Preliminary results indicate a HER2/c-Src complex in a cell line which overexpresses both tyrosine kinases, yet no complex is seen in a cell line expressing c-Src alone. Cell lines generated will be tested for tumorigenicity in assays of [^{3}H]-thymidine incorporation, soft agar growth, and tumor formation in nude mice. These studies are designed to identify potential mechanisms of action between HER family members and c-Src, and target molecules active in breast cancer formation.

4. Pathways to Cancer, Cold Spring Harbor Abstract, 1998

INTERACTIONS BETWEEN C-SRC AND HER2: INVOLVEMENT IN HUMAN BREAST TUMOR FORMATION. Allison P.Belsches-Jablonski, J.S.Biscardi, D. Peavy, S.J.Parsons. Dept. of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA 22908

Previous studies by several laboratories have shown that functional interaction between members of the human EGF receptor (HER) receptor tyrosine kinase family, and c-Src, a non-receptor tyrosine kinase, may be involved in breast tumor formation. Our laboratory has demonstrated that overexpression of HER1 and c-Src, in C3H10T1/2 murine fibroblasts, results in an EGF-dependent synergistic increase in tumor formation in nude mice. This increased tumorigenicity correlates with the formation of an *in vivo* c-Src/HER1 complex, two novel phosphorylation sites on HER1, and enhanced phosphorylation of receptor substrates, suggesting association of HER1 with c-Src causes hyperactivation of receptor kinase activity, leading to enhanced intracellular signaling and augmented growth. Overexpression of HER2 occurs in approximately 30% of human breast cancers, and is correlated with poor patient prognoses. Elevated levels and/or activity of c-Src have been demonstrated in human breast cancers. Because of HER1 and HER2 structural homology and elevated expression in human breast cancers, we propose HER1 and HER2 interact with c-Src by similar mechanisms to augment cellular growth and tumor progression. To examine potential c-Src/HER2 interactions, we studied human breast tumor tissue, and a panel of human breast carcinoma cell lines for expression of HER family members and c-Src by Western blotting. In almost all samples, either HER1 or HER2 is overexpressed, but not both simultaneously. Lysates from tumors and cell lines were also tested for heterocomplexes containing c-Src and HER2. Three of thirteen tumor tissues and three of nine human breast tumor cell lines which overexpress both HER2 and c-Src exhibit constitutive *in vivo* c-Src/HER2 heterocomplexes. In two of the breast carcinoma cell lines which exhibit the c-Src/HER2 complex, heregulin treatment, but not EGF, results in augmented growth, as assayed by cell counting and colony formation in soft agar. In contrast, none of the cell lines tested (3) which were negative for HER2/c-Src association responded to heregulin. These data demonstrated correlation between complex formation and sensitivity to heregulin, perhaps mediated through HER2 and c-Src.

5. "Specificity in Signaling", Keystone Symposium, Keystone, CO, April 1999.

INVESTIGATION OF THE ROLE OF P190RHOGAP IN EGF-INDUCED ACTIN CYTOSKELETAL DYNAMICS. M. D. Haskell.*, B. D. Dukes, A. L. Nickles, S. J. Parsons. Dept. of Microbiology, University of Virginia, Charlottesville, VA..

P190 RhoGAP is a 190kDa phosphoprotein that contains an N-terminal GTP-binding domain, a middle domain, and a C-terminal GTPase activating protein (GAP) domain that is specific for the Rho family of small GTPases. Rho regulates several cytoskeleton-coordinated events in the cell, such as actin stress fiber dynamics following growth factor stimulation. One function of p190 may be to regulate actin cytoskeletal dynamics via hydrolysis of GTP-bound Rho. Studies in our laboratory in C3H10T1/2 murine fibroblasts have demonstrated that p190 is a preferred substrate c-Src and that c-Src-mediated tyrosine phosphorylation of p190 on Y1105 is associated with an acceleration of actin cytoskeletal rearrangements following EGF stimulation. We generated six GST bacterial fusion protein constructs of p190 to identify which domains of the molecule are required for regulation of the actin cytoskeleton: wild type (*wt*) GAP

domain (aa1261-1469), Y1283F and Y1283D mutants of the GAP domain, *wt* middle domain (aa380-1180), *wt* middle domain/GAP domain (aa380-1469), and *wt* section 3 of the middle domain construct (aa919-1180). These constructs were microinjected into C3H10T1/2 murine fibroblasts and then stimulated with EGF over a 30 min time course. Preliminary data shows that injection of *wt* GAP domain causes a dramatic, EGF-independent disassembly of actin stress fibers in these cells. However, when either the Y1283F or Y1283D mutants were injected, there was no actin dissolution observed above the control GST-injected levels. *In vitro,* Rho GTPase assays demonstrated that the GAP activity of the mutants was severely diminished when compared to the GAP activity of *wt* RhoGAP domain. Furthermore, injection of the middle domain/GAP construct in Neo cells restored EGF regulation of the RhoGAP activity and partially reduced the extent of actin dissolution seen in normal controls. These results indicate that the middle domain may negatively regulate the activity of the GAP domain, and thus the ability of p190 to regulate cytoskeletal changes.

6. Oncogene Meeting, 1999 *Note 2 Abstracts

Abstract 1

SRC FAMILY KINASES AND HEREGULIN COOPERATE TO PROMOTE HUMAN BREAST CANCER CELL GROWTH AND SURVIVAL. Allison P. Belsches-Jablonski, Jacqueline S. Biscardi, David A. Tice, Davis A. Romney, Dena R. Peavy and Sarah J. Parsons* Department of Microbiology and Cancer Center, Box 441, University of
Virginia Health Sciences Center, Charlottesville, VA 22908

Evidence from a murine fibroblast model and human breast cancer cells indicates that c-Src and the human EGF receptor (HER1) synergize to enhance neoplastic transformation and progression of mammary epithelial cells. To investigate whether interactions between c-Src and other HER family members may also play a role in breast tumor progression, we characterized a panel of thirteen human breast carcinoma cell lines and thirteen tumor samples for expression levels of HER family members and c-Src and examined a subset of the cell lines for their dependency on Src family members for heregulin (HRG)-augmented, anchorage-dependent growth in reduced serum or colony formation in soft agar. By Western immunoblotting, we found that (1) all cell lines overexpressed one or more HER family member, (2) ~60% co-overexpressed HER2/*neu* and HER3, (3) HER1 was rarely co-overexpressed with HER2/HER3, and (4) HER4 was overexpressed in only 30% of the cases. c-Src was overexpressed in 70% of the samples. 90% of the tumor tissues overexpressed HER2, while 64% overexpressed c-Src. Growth in low serum was potentiated by HRG in five of six cell lines tested (irrespective of HER2/3 or c-Src levels), and colony formation in soft agar was enhanced in three of the six. This latter response was observed only in those cell lines that exhibited a c-Src/HER2 heterocomplex, suggesting that physical association between c-Src and HER2 may facilitate HRG-potentiated, anchorage-independent growth. HRG effects on both colony formation and growth in reduced serum were either partially or completely ablated by PP1, a Src family kinase inhibitor. In addition, long-term treatment of adherent cells with PP1 induced apoptosis in all cell lines tested, a process that was significantly reversed by HRG treatment. These data provide the first functional evidence for co-operativity between HRG and Src family kinases in the survival and growth of human breast tumor cells and reveal that the co-operativity is independent of the levels of either HER family members or c-Src.

Abstract 2

INVESTIGATION OF THE ROLE OF P190RHOGAP IN EGF-INDUCED ACTIN CYTOSKELETAL DYNAMICS. M. D. Haskell.*, B. D. Dukes, A. L. Nickles, S. J. Parsons. Dept. of Microbiology, University of Virginia, Charlottesville, VA..

P190 RhoGAP is a 190kDa phosphoprotein that contains an N-terminal GTP-binding domain, a middle domain, and a C-terminal GTPase activating protein (GAP) domain that is specific for the Rho family of small GTPases. Rho regulates several cytoskeleton-coordinated events in the cell, such as actin stress fiber dynamics following growth factor stimulation. One function of p190 may be to regulate actin cytoskeletal dynamics via hydrolysis of GTP-bound Rho. Studies in our laboratory in C3H10T1/2 murine fibroblasts have demonstrated that p190 is a preferred substrate c-Src and that c-Src-mediated tyrosine phosphorylation of p190 on Y1105 is associated with an acceleration of actin cytoskeletal rearrangements following EGF stimulation. We generated six GST bacterial fusion protein constructs of p190 to identify which domains of the molecule are required for regulation of the actin cytoskeleton: wild type (*wt*) GAP domain (aa1261-1469), Y1283F and Y1283D mutants of the GAP domain, *wt* middle domain (aa380-1180), *wt* middle domain/GAP domain (aa380-1469), and *wt* section 3 of the middle domain construct (aa919-1180). These constructs were microinjected into C3H10T1/2 murine fibroblasts and then stimulated with EGF over a 30 min time course. Preliminary data shows that injection of *wt* GAP domain causes a dramatic, EGF-independent disassembly of actin stress fibers in these cells. However, when either the Y1283F or Y1283D mutants were injected, there was no actin dissolution observed above the control GST-injected levels. *In vitro,* Rho GTPase assays demonstrated that the GAP activity of the mutants was severely diminished when compared to the GAP activity of *wt* RhoGAP domain. Furthermore, injection of the middle domain/GAP construct in Neo cells restored EGF regulation of the RhoGAP activity and partially reduced the extent of actin dissolution seen in normal controls. These results indicate that the middle domain may negatively regulate the activity of the GAP domain, and thus the ability of p190 to regulate cytoskeletal changes.

- List of Personnel: Allison P. Belsches-Jablonski
Michelle D. Haskell
Sarah J. Parsons