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Compensatory ErbB3/c-Src signaling enhances carcinoma cell survival to ionizing radiation

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Summary

EGFR and ErbB2 are two members of the ErbB family of receptor Tyr Kinases identified as therapeutic targets for treating carcinomas. Breast carcinoma cells express different complements and variable proportions of ErbB receptor Tyr kinases, which activate unique and redundant signaling cascades that are essential for cell survival. Previously it was shown that a COOH-terminal truncation mutant of the EGFR (EGFR-CD533) blocks EGFR dependent signals and radiosensitizes breast carcinoma cells. In this study the effects of EGFR-CD533 and an analogous truncation mutant of ErbB2 (ErbB2-CD572) on ErbB receptor family dimerization and signaling are further investigated. Using adenoviral vectors in breast carcinoma cell lines with variable ErbB expression profiles, we demonstrate different effects for each deletion mutant. EGFR-CD533 blocks ligand stimulation of EGFR, ErbB2, and ErbB4, but is associated with a compensatory Tyr kinase activity resulting in phosphorylation of ErbB3. In contrast, ErbB2-CD572 produces a weaker, non-specific pattern of ErbB receptor family inhibition, based upon the ErbB expression pattern of the cell type. Investigation of the compensatory Tyr kinase activity associated with EGFR-CD533 expression identified an ErbB3/c-Src signaling pathway that regulates expression of anti-apoptotic Bcl family proteins. This signaling is active in the T47D cell line, which inherently over-express ErbB3, absent in MDA-MB231 cells, which have low ErbB3 expression levels, and is restored in a MDA-MB231 cell line engineered to over-express ErbB3. Furthermore we demonstrate that ErbB3/c-Src signaling is radioprotective, and that its elimination through pharmacologic inhibition of c-Src enhances radiation-induced apoptosis. In summary, these studies identify a novel ErbB3/c-Src survival signal and point to ErbB3 expression levels as an important variable in therapeutic targeting of ErbB receptors in breast carcinoma cells.

Introduction

Autocrine growth regulation of carcinoma cells by the ErbB family of receptors (EGFR, ErbB2, ErbB3, and Erbb4) is implicated as a mechanism for both cellular proliferation and protection from programmed cell death. Clinically, EGFR or ErbB2 expression is a negative prognostic indicator for multiple carcinoma types [1,2], and in experimental systems, activation of intrinsic ErbB Tyr kinase activity produces neoplastic transformation [3]. ErbB receptors propagate signals laterally, through the formation of homo- and hetero-oligomeric complexes, resulting in the diversification and amplification of downstream signaling [4,5]. Most carcinomas express multiple ErbB receptor types, and the spectrum of ErbB receptors expressed by a given tumor cell determines the recruitment of either proliferative or anti-apoptotic signal transduction cassettes.

All members of the ErbB receptor family contain a Cys-rich extracellular domain, a hydrophobic transmembrane domain, and a COOH-terminal intracellular kinase domain proximal to Tyr phosphorylation sites. However, each receptor also possesses unique characteristics that affect receptor activity and function. EGFR and ErbB4 are fully functional receptors with binding sites for EGF and HRG family ligands, respectively, as well as functional Tyr kinase domains. ErbB2 has a functional Tyr kinase domain, but no identified ligand, and is activated through interaction with other ErbB receptors [6] or through enhanced expression [7]. ErbB3 also binds HRG family ligands, but lacks an active kinase domain [8]. ErbB3 signaling is therefore dependent upon recruitment of other Tyr kinases [9,10]. Despite these differences, ligand stimulated ErbB receptors share

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a common activation mechanism that is initiated by ligand binding, depends upon conformational change and dimer/oligomer stabilization, and produces kinase activation and receptor trans-phosphorylation. The structural principles underlying this mechanism have recently been defined using X-ray crystallography and provide further understanding of ErbB receptor homo- and hetero-dimerizations [11–16].

Functional inhibition of ErbB receptors has been studied using ErbB receptor COOH-terminal deletion (CD) mutants. These truncated receptors lack the intracellular kinase domain and form inactive dimmers with wild type recptors via extracellular domain dimerization [17–20]. In experimental systems an EGFR deletion mutant (EGFR-CD533) has been shown to block stimulation of either EGFR or ErbB2 [21] and similar results have been reported for ErbB2 and Neu deletion mutants [22]. Because wild type ErbB receptors have distinct homo/hetero-dimerization and signaling patterns, EGFR and ErbB2 deletion mutants are expected to produce differential inhibition within complex ErbB receptor networks. However a comparison of these ErbB deletion mutants in carcinoma cells with diverse ErbB receptor expression profiles has not been performed, and their inhibitory effect on HRG-stimulated ErbB2/ErbB3 activation is unknown. Considering the importance of receptor dimerization for ErbB receptor tyrosine kinase activity, studies with these truncation mutants in the context of different ErbB receptor expression profiles is likely to uncover important mechanistic insights on ErbB receptor network signaling.

Investigation of the ErbB receptor network signaling has demonstrated that initiation of cellular programs is dependent upon the ErbB receptor complement expressed by a given cell [3,6]. This concept is supported by studies of radiation-induced ErbB receptor activation that have identified both proliferative and anti-apoptotic signals that are determined by specific ErbB receptors [23,24]. In these experiments selective pharmacologic inhibition of a single ErbB receptor type, such as EGFR or ErbB2, did not block parallel, downstream signals generated by other ErbB receptors co-expressed by the cell. Results using peptide growth factors are similar and show that blockade of a single ErbB receptor type inhibits cell proliferation, but that proliferation is restored by ligand-induced activation of other ErbB receptor species [25]. In summary, these studies demonstrate a high degree of ErbB network coordination and interaction that functions to sustain growth promoting signals in carcinoma cells.

ErbB receptors also engage in protein–protein interactions with non-receptor Tyr kinases. In breast carcinoma cells c-Src phosphorylates the kinase domain of the EGFR, enhancing the receptor's catalytic activity [26]. Src also associates with ErbB2 in breast carcinoma cell lines and tumor samples [27]. These interactions between c-Src and EGFR/ErbB2 are linked to enhanced mitogenesis for the EGFR, anchorage independent cell growth for ErbB2, and anti-apoptotic responses for both EGFR and ErbB2 [26,28]. While ErbB2 is implicated as one mediator of HRG-induced activation of c-Src [29], interactions of ErbB3 and ErbB4 with c-Src have not yet been defined.

In the present study we evaluate ErbB receptor network signaling by comparing the effects of expressing EGFR and ErbB2 deletion mutants on EGF and HRG β directed signaling in breast carcinoma cells that express heterogeneous complements of ErbB receptors. These experiments demonstrate that the relative inhibitory activities of the deletion mutants are dependent on the ErbB expression profile of a cell, and reveal a novel ErbB3/c-Src anti-apoptotic signal that enhances carcinoma cell survival after exposure to ionizing radiation.

Materials and methods

Reagents

Unless otherwise stated all reagents were from Sigma Chemical Co. (St Louis, MO). The fetal bovine serum (FBS) was from Hyclone (Logan, Utah). Antibodies for C-terminal immune precipitation of the EGFR (Ab-15), ErbB2 (Ab-8), ErbB3 (Ab-4), and ErbB4 (Ab-1), as well as antibodies for western blots of EGFR (Ab-14), ErbB2 (Ab-3), ErbB3 (Ab-7), and ErbB4 (Ab-4) were supplied by Neomarkers. Antibodies specific for phosphotyrosine (No.2, No. 102) were purchased from Calbiochem (San Diego, CA) or Cell Signaling (Beverly, Mass.). Phosphorylation site specific antibodies to Tyr 416 of c-Src, were supplied by Cell Signaling. The antibodies recognizing c-Src (N-16), actin (C-2), Bcl-XL (S-18) and Mcl-1 (S-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling and Promega (Madison, WI) respectively. Protein A/G agarose beads from Calbiochem were used for primary antibody precipitation in immune complex assays. Pharmacologic kinase inhibitors (PP2, AG825, and AG490) were all purchased from Calbiochem.

Vectors

The ErbB2 cytoplasmic deletion mutant (ErbB2-CD572) is a truncated form of c-ErbB2 that lacks 572 amino acids of the cytoplasmic domain of the WT receptor. This cDNA was constructed by isolating an *Xba*I/*Acc*III fragment from the pCHC-ErbB2 plasmid (a gift from Dr. F. Kern) and ligating it into the *Xba*I/*Eco*RV site of pZero2.1. The ErbB2 fragment was then cloned into the pZTG-CMV adenoviral transfer vector using *Xba*I/*Hin*dIII sites. The Ad-LacZ and Ad-CD533 vectors have been described previously and the Ad-ErbB2-CD572 was produced according to these methods [22]. The ErbB3 expression vector was generated

using an ErbB3 cDNA (a gift from Dr. S. Aaronson, Mount Sinai School of Medicine, N.Y.), isolated by digestion with SalI and cloned into the *Xho*I site of pZero2.1. Following *Xba*I/*Kpn*I digestion, the full length ErbB3 cDNA was directionally cloned into a multiple cloning site modified pCMV vector (pTAG vector; Stratagene; Austin, Tex.). The WT c-Src, DN c-Src, and empty vector plasmids were gifts from Dr. S. Parsons (University of Virginia).

Generation and treatment of cell lines

The MDA-MB231 and T47D mammary carcinoma cell lines were purchased from the American Type Tissue Collection (ATCC; Rockville, MD). The CHO-ErbB3 and the MDA-ErbB3 cell lines were isolated following transfection of the parent cell line with the pCMV-ErbB3 plasmid and selection for clones with 800 μg/ml G418. Cell lines were grown as monolayers at 37 °C in RPMI 1640 medium plus 5% FBS and antibiotics (penicillin/streptomycin). Adenoviral infection was performed 3 days after plating, and experimental manipulation was performed 48 h after viral transduction. Except for experiments where apoptosis was measured, the culture media was aspirated and replaced with RPMI plus 0.5% FCS and antibiotics 18 h prior to experimentation.

For studies involving pharmacologic kinase inhibitors and Tyr phosphorylation analysis, cells were pretreated for 1 h prior to experimentation with either 5 μM AG825, 10 μM PP2, or 20 μM AG490. The PP2 concentration was determined by dose response experiments (data not shown) and similar to previously reported inhibitory concentrations [28]. PP2 did not block EGF stimulation of EGFR or downstream activities of PI3K/Akt and MAPK/p90S6K (data not shown). For apoptosis and Bcl expression studies, cells were exposed to PP2 for 24 h prior to experimentation. Growth factor stimulation of ErbB receptors was performed for 5 min with 10 ng/ml of either EGF or HRG β. Following growth factor stimulation, cells were washed with ice cold PBS and snap frozen on dry ice for further processing.

Adenoviral infection

Replication incompetent adenovirus was generated by established techniques [30]. Transduction of carcinoma cells with Ad-LacZ as a control, Ad-ErbB2-CD572, or Ad-EGFR-CD533 was performed at an MOI of 10 for T47D cells and an MOI of 50 for MDA cells. The MOI for both cell lines were optimized for maximum infection [31]. Cell lines were grown in 6 cm dishes to approximately 60% confluence prior to infection. The adenovirus was diluted in sterile PBS, added to 1 ml of pre-conditioned media from each dish, and then incubated with agitation for 4 h. After incubation, the virus containing media was aspirated and replaced with fresh media.

Measurement of apoptosis

The percentage of apoptotic cells was determined 24 h following exposure to 8 Gy from a ^{60}Co source at a dose rate of 1.8 Gy per minute. Floating cells were collected along with trypsinized adherent cells by centrifugation. Cell pellets were resusupended in methanol:acetic acid (3:1), centrifuged, and resusupended in methanol:acetic acid twice more. The fixed cells were then pipetted onto glass microscope slides and allowed to air dry. Following Giemsa staining, an average of 1600 cells per slide were counted to determine the percentage of apoptotic cells.

Immunologic assays

Immunoprecipitation and Western blot analysis were performed as described previously [23].

Statistics

All experiments were performed at least in triplicate. Averages are reported with the standard error of the mean (SEM). The student's t test was applied to determine p values and statistical significance.

Results

Genetic strategies for inhibition of ErbB signaling

Expression of ErbB deletion mutants disrupts ErbB dependent signaling through inhibitory homo- and hetero-dimerizations [17]. Since wild type (WT) EGFR and ErbB2 have distinct homo- and hetero-dimerization patterns, deletion mutants of each receptor may show different inhibitory profiles depending on the ErbB receptor composition of a given cell [24]. We investigated these effects by comparing two analogous COOH-truncation mutants. EGFR-CD533 and ErbB2-CD572 mutants retain the extracellular and transmembrane domains of the WT receptor but have cytoplasmic COOH terminal deletions of 533 and 572 amino acids respectively. As a consequence they are kinase dead and are missing all known tyrosine phosphorylation sites. Expression of each mutant was accomplished by infecting cells with a replication incompetent adenoviral vector encoding either mutant. Protein expression of each mutant 48 h post-infection is ≥20 fold higher than the endogenous, WT ErbB receptor and has no measurable effects on WT ErbB expression levels (Figure 1).

Inhibition of growth factor stimulated ErbB receptors

The T47D and MDA-MB231 mammary carcinoma cells differ in their ErbB family receptor expression patterns [31,32]. MDA-MB231 cells have high and moderate levels of EGFR and ErbB2, respectively, low levels of ErbB3, and no detectable ErbB4 expression. In contrast, T47D

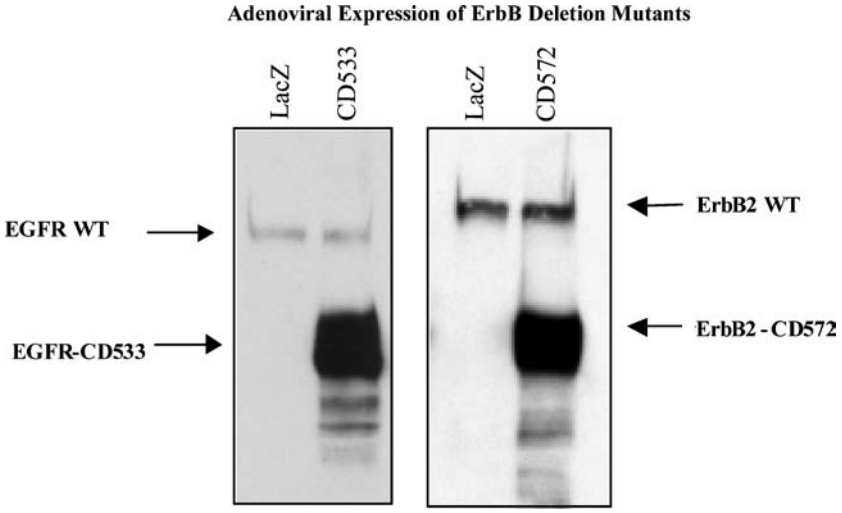


Figure 1. Protein expression of EGFR and ErbB2 COOH-terminal deletion (CD) mutants (EGFR-CD533 and ErbB2-CD572). Each mutant contains the extracellular and transmembrane domains, but lacks the intracellular kinase domain and c-terminal phosphorylation sites. Viral transduction of the deletion mutants is shown in MDA-MB231 cells for EGFR-C533 and in T47D cells for the ErbB2-CD572.

cells express very low levels of EGFR and relatively higher levels of ErbB2, ErbB3, and ErbB4. These differing expression profiles determine the biologic response of each cell line to EGF and HRG β stimulation (Figure 2). Thus, EGF activates Tyr phosphorylation of the EGFR and ErbB2, but not ErbB3 or ErbB4, in both T47D and MDA-MB231 cells. In T47D cells, which express ErbB3 and ErbB4 at relatively high levels, HRG β is a strong activator of ErbB2, ErbB3, and ErbB4. Since ErbB2 does not bind HRG β or EGF, its activation is consistent with its trans-activation by EGFR or ErbB3/ErbB4.

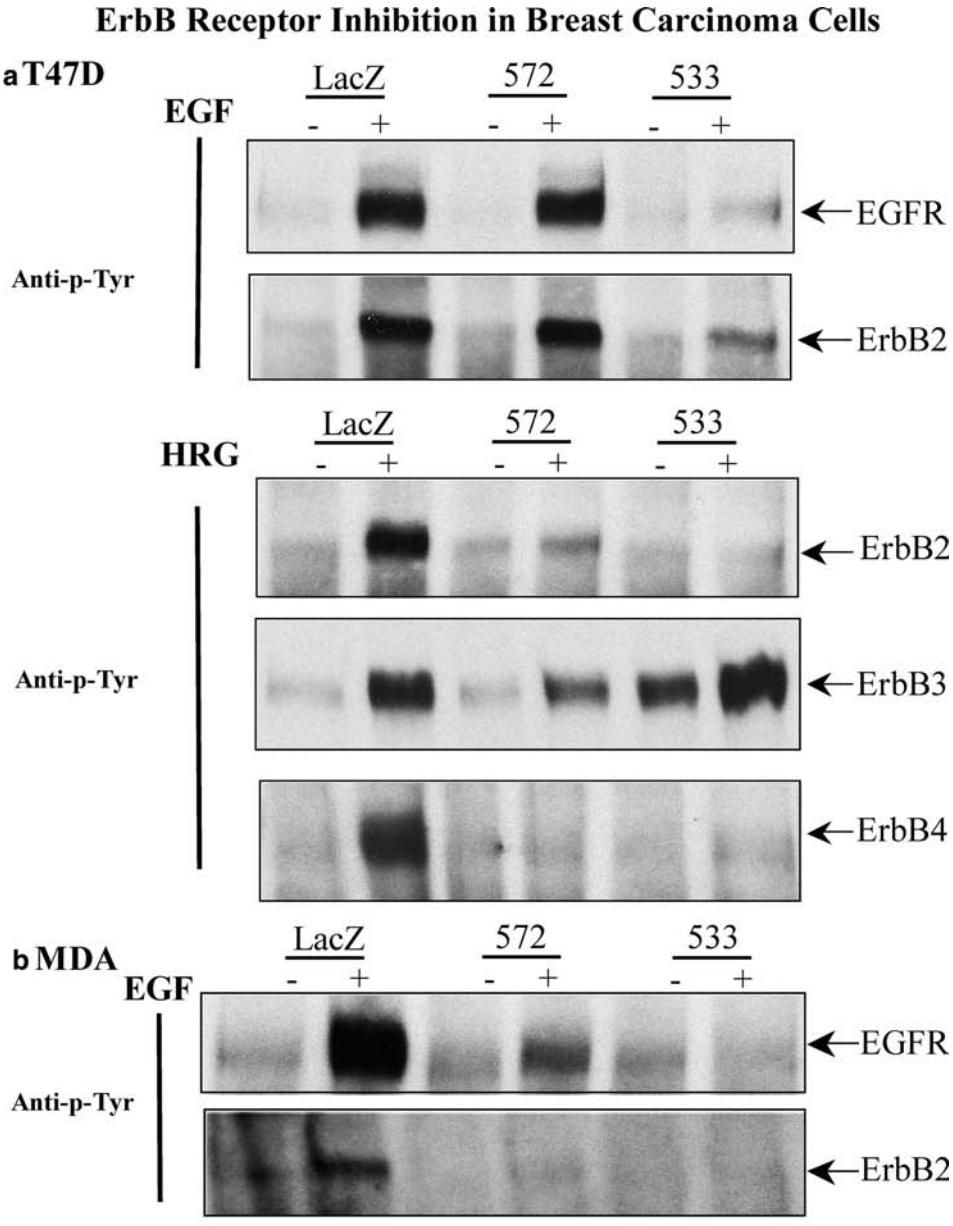
Using these defined activation profiles for T47D and MDA-MB231 cells, we compared the effects of expressing either EGFR-CD533 or ErbB2-CD572 deletion mutants on ErbB receptor activation. Activation was measured by immune precipitation of the receptor followed by quantification of Western blot Tyr phosphorylation. Representative blots are shown in Figure 2a and the averaged results from several experiments are summarized in Figure 2c. In T47D cells EGFR-CD533, but not ErbB2-CD572, significantly blocks EGF-induced EGFR and ErbB2 phosphorylation (Figure 2a). EGFR-CD533 expression in T47D cells also inhibits HRG β-stimulated Tyr phosphorylation of ErbB2 and ErbB4, however this is accompanied by an unexpected enhancement of basal ErbB3 phosphorylation. In comparison ErbB2-CD572 has a different inhibitory profile, and only partially inhibits HRG β-stimulation of ErbB2 and ErbB3 while completely blocking ErbB4 Tyr phosphorylation. In the MDA-MB231 cells expression of either EGFR-CD533 or ErbB2-CD572 blocks EGF-induced EGFR and ErbB2 Tyr phosphorylation. HRG β fails to activate ErbB receptor phosphorylation in MDA-MB231 cells, consistent with the low or absent expression of ErbB3 and ErbB4, respectively, in this cell line.

The results demonstrate distinct ErbB family inhibitory patterns for each ErbB deletion mutant. The EGFR-CD533 potently blocks EGF-stimulated EGFR and ErbB2 phosphorylation independent of the ErbB expression patterns of the cell. This inhibitory effect can be explained by EGFR blockade alone, as ErbB2 is not a receptor for EGF. However, the additional inhibition of HRG β-stimulated ErbB2 and ErbB4 in T47D cells demonstrates that EGFR-CD533 engages in inhibitory hetero-dimerizations with these ErbB family members. These results are in contrast to those obtained with the ErbB2-CD572. The ErbB2 deletion mutant is only a potent EGFR inhibitor in MDA-MB231 and not T47D cells. Because T47D cells express much higher levels of ErbB2 and ErbB3 and relatively low levels of EGFR, these results are consistent with inhibition through mass action. Accordingly, in T47D cells the inhibitory effects of ErbB2 CD572 are seen on HRG-induced signaling instead of EGF-induced signaling. Thus experiments with T47D and MDA-MB231 breast carcinoma cells demonstrate that inhibitory effects of ErbB2-CD572 are dependent on the ErbB expression profile of the cell. These results are consistent with the current understanding of roles for ErbB receptor extracellular domains in dimerization (see Discussion).

The simultaneous analysis of ErbB receptor activation profiles in T47D cells revealed a significant increase in basal ErbB3 phosphorylation following EGFR-CD533 expression. This phosphorylation occurs despite potent inhibition of EGFR, ErbB2, and ErbB4. Because ErbB3 has an inactive kinase domain, it's phosphorylation indicates enhanced activity of another category of Tyr kinases. This response is not observed with ErbB2-CD572 expression and is possibly a compensatory response to potent inhibition of the other members of the ErbB receptor network.

Compensatory ErbB3 phosphorylation is mediated by c-Src family tyrosine kinases

An enhancement of basal tyrosine kinase activity, as measured by ErbB3 Tyr phosphorylation, suggests an



c **Average ErbB p-Tyr inhibition**

		EGF			HRG		
		LacZ	CD572	CD533	LacZ	CD572	CD533
MDA	EGFR	1.0	0.19±3*	0.05±3*	n/a		
	ErbB2	1.0	0.07±2*	0.07±2*	n/a		
T47D	EGFR	1.0	0.99±1	0.20±8*	n/a		
	ErBB2	1.0	0.95±3	0.42±5*	1.0	0.39±5*	0.10±5*
	ErbB3	n/a			1.0	0.47±10*	1.0±9
	ErbB4	n/a			1.0	<0.05 *	<0.05 *

Figure 2. ErbB receptor inhibition in mammary carcinoma cells. ErbB receptor phosphorylation was determined by Western blot analysis following a 5 min exposure to 10 ng/ml of either EGF or HRG. (a) T47D cells were transduced with adenovirus expressing either LacZ, EGFR-CD533, or ErbB2-CD572. Western blots for phospho-Tyr were performed after immunoprecipitation of specific ErbB receptors. Blots were stripped and reprobed with specific ErbB receptor antibodies to establish equal loading (data not shown). (b) Inhibition of ErbB receptors in MDA-MB231 cells by EGFR-CD533 and ErbB2-CD572. (c) Average values for ErbB receptor inhibition following quantification from at least 3 independent experiments. An * indicates significant inhibition from control LacZ infected cells.

EGFR and ErbB2 independent signal that is potentially cytoprotective. As such, this Tyr kinase signaling represents an important resistance mechanism to EGFR inhibition. However, this Tyr kinase activity does not simply replace EGFR and ErbB2 function, as EGFR-CD533 virtually eliminates MAPK activity (data not shown). To identify the source of this enhanced Tyr kinase signaling, EGFR-CD533 infected T47D cells were treated with specific pharmacologic inhibitors for JAK, (AG490), ErbB2 (AG825), and c-Src (PP2). The results show that neither AG825 nor AG490 eliminates ErbB3 Tyr phosphorylation (Figure 3). However 1 h pretreatment with PP2, an inhibitor of Src family Tyr kinases, blocks this compensatory phosphorylation at a dose of 10 μM. This data suggests that c-Src is the kinase that phosphorylates ErbB3.

Activation of c-Src in mammary carcinoma cells

The functional relationship between ErbB receptors and c-Src was further established by examining basal c-Src phosphorylation at Tyr 416, a kinase domain autophosphorylation site that correlates with activity [33]. In T47D cells, EGFR-CD533 expression does not block c-Src activation confirming that its phosphorylation is independent of EGFR/ErbB2 activity (Figure 4). In contrast, EGFR-CD533 expression in MDA-MB231 cells does block Tyr 416 phosphorylation, demonstrating dependence on EGFR and/or ErbB2 activity. The difference between c-Src phosphorylation states in T47D and MDA-MB231 cells suggests that ErbB3 may play a role in regulating c-Src activity. To test this hypothesis MDA-MB231 cells that stably over-express ErbB3 (MDA-ErbB3) without changes to EGFR and ErbB2 expression levels were generated as described in Materials and Methods. In this cell line, the effect of EGFR-CD533 expression on Src Tyr 416 phosphorylation was reversed from the parent MDA-MB231 cell line, and there was no inhibition of Src activation. Together these experiments suggest that Src phosphorylation is regulated by EGFR/ErbB2 receptor activity, except in the presence of high levels of ErbB3. Thus in T47D and MDA-ErbB3 cells an ErbB3 dependent c-Src phosphorylation mechanism appears operative.

Src dependent anti-apoptotic signaling

Src signaling regulates the expression of mitochondrial proteins involved in apoptosis [28,34]. Because EGFR-CD533 expression has differential effects on c-Src activity, we measured its effect on the expression of anti-apoptotic proteins, Mcl-1 and Bcl-XL, in each breast carcinoma cell line (Figure 5). In T47D and MDA-ErbB3 cells Mcl-1 or Bcl-XL expression, respectively, was insensitive to infection with Ad-EGFR-CD533. MCL-1 or Bcl-XL expression was, however, inhibited with the c-SRC inhibitor, PP2. In contrast, EGFR-CD533 and PP2 were equally effective in blocking Bcl-XL expression in MDA-MB231 cells. These results for ErbB-dependent modulation of anti-apoptotic protein expression are in agreement with the findings in Figure 4 for EGFR-CD533 expression and c-Src activation. In summary the inhibition of Bcl family protein expression in all three breast carcinoma cell lines corresponds directly to the inhibition of c-Src signaling. Because EGFR-CD533 does not block c-Src or Bcl family protein expression in the context of enhanced ErbB3 expression, the results suggest that high expression levels of ErbB3 provide a Src dependent, anti-apoptotic signal.

ErbB3/c-Src signaling reduces cellular apoptosis

To evaluate the protective role of ErbB/c-Src signaling in cell survival, we compared the apoptotic responses of each breast carcinoma cell line following exposure to ionizing radiation (Figure 6). In T47D cells EGFR-CD533 expression enhances basal apoptosis as compared to LacZ controls, but does not significantly enhance apoptosis with either radiation or PP2 treatment alone. However, a significant enhancement of apoptosis is observed following radiation in combination with EGFR-CD533 expression and c-Src inhibition ($p < 0.01$). In MDA-MB231 cells EGFR-CD533 not only enhances basal apoptosis over LacZ controls, but

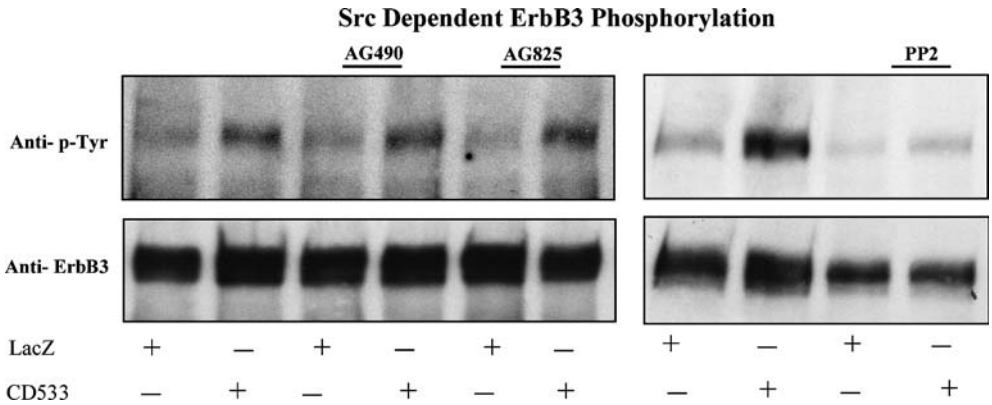


Figure 3. Compensatory ErbB3 phosphorylation is blocked by Src inhibition with PP2. Following expression of LacZ or EGFR-CD533, cells were treated with tyrosine kinase inhibitors AG490 (20 μM), AG825 (5 μM), or PP2 (10 μM) for 1 h. ErbB3 was then immunoprecipitated and Tyr phosphorylation was determined by western blot analysis. Blots are representative of at least three independent experiments.

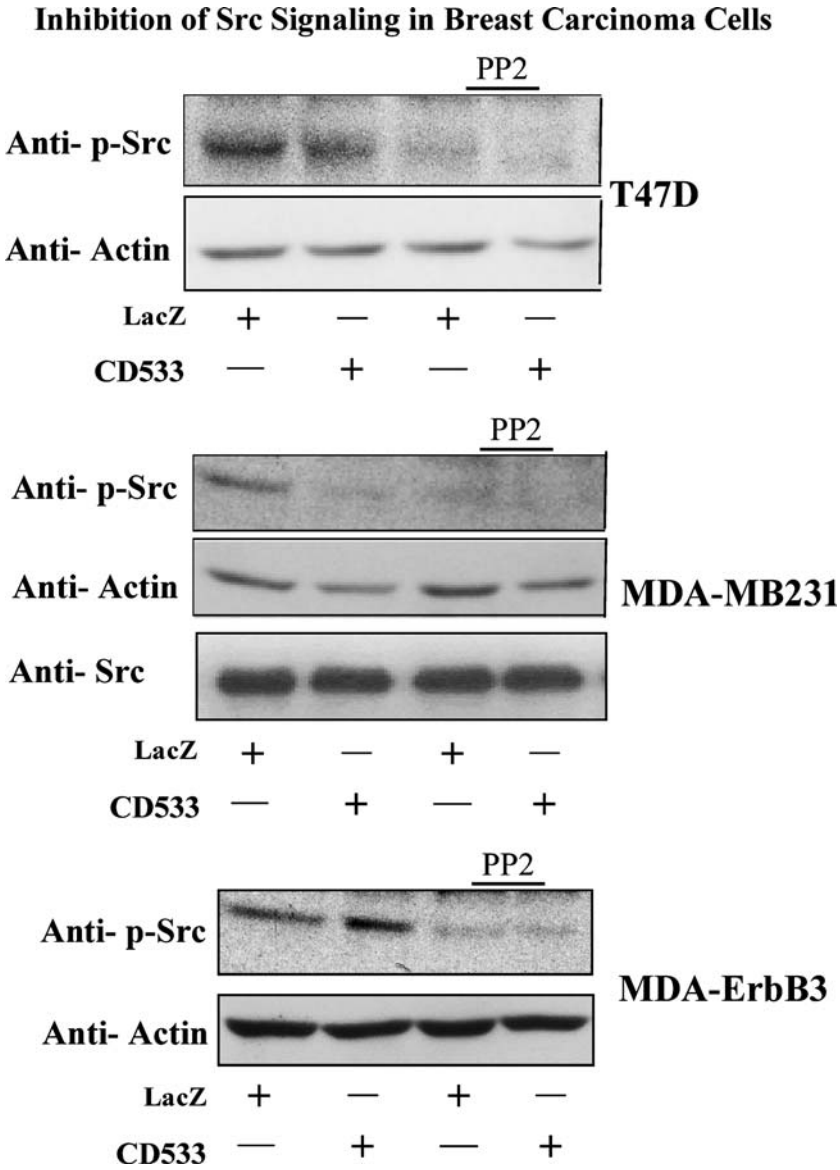


Figure 4. Inhibition of Src signaling in breast carcinoma cells. Breast carcinoma cells expressing LacZ or EGFR-CD533 were evaluated for basal phosphorylation of Src Tyr 416 phosphorylation by western blot with or without pretreatment with 10 μM PP2. Blots were reprobed for actin as a loading control. For MDA-MB231 cells, parallel blots were probed for total Src to determine the effect of EGFR-CD533 on Src expression.

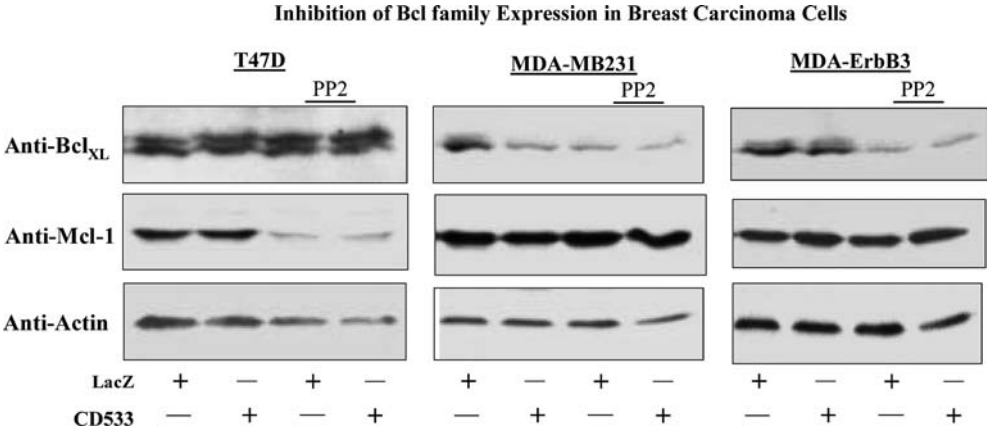


Figure 5. Expression of anti-apoptotic proteins is dependent on Src activity in breast carcinoma cells. Western blots of Bcl-XL and Mcl-1 protein expression are shown for breast carcinoma cells following LacZ or EGFR-CD533 expression and in the presence or absence of pretreatment with 10 μM PP2. Blots were stripped and reprobed for actin to insure equal loading. Western blots are representative of results for three independent experiments.

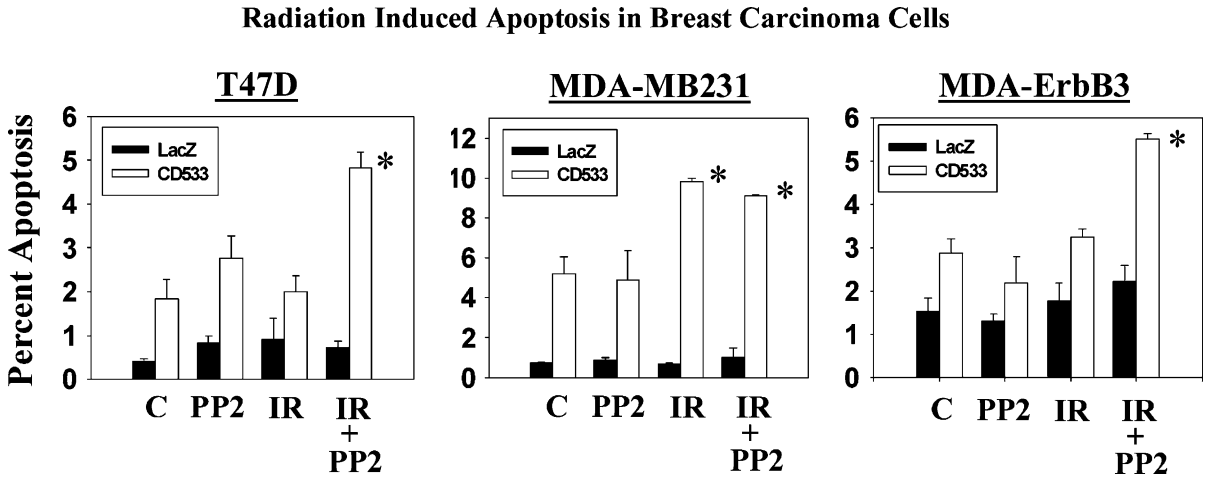


Figure 6. Apoptosis in breast carcinoma cells. Following transduction with either LacZ or EGFR-CD533 expressing virus, breast carcinoma cells were exposed to the following conditions: no treatment, 10 μM PP2 only, 8 Gy, or 1 h pretreatment with PP2 followed by 8Gy. 24 h later cells were harvested and fixed as described in Materials and Methods to determine the percentage of apoptosis. Each graph illustrates the average of three independent experiments and error bars represent the standard error of the mean (SEM). An (*) represents a statistically significant difference ($p < 0.01$) from control, non-irradiated EGFR-CD533 expressing samples.

also combines with radiation to significantly enhance apoptosis ($p < 0.01$). However, in this cell line pretreatment with PP2 has no additional effect on apoptosis, consistent with the experimental evidence that c-Src activity is already blocked by EGFR-CD533 expression. In contrast the MDA-ErbB3 cells behave like T47D cells in these experiments. EGFR-CD533 does not enhance radiation-induced apoptosis in MDA-ErbB3 cells, and like T47D cells requires Src inhibition with PP2 to significantly enhance apoptosis ($p < 0.01$). These findings parallel those obtained with both Src Tyr phosphorylation and Bcl family protein expression, providing evidence for radio-protective ErbB3/c-Src signaling in breast carcinoma cells.

ErbB3/c-Src interactions

For a more detailed description of interactions between c-Src and ErbB3, CHO cells with stable expression of ErbB3 (CHO-ErbB3) were generated and transfected with either a WT c-Src or a DN mutant of c-Src. Both WT and DN c-Srcs form stable complexes with ErbB3 as assessed by immune precipitation (Figure 7), however only the kinase active, WT c-Src mediates ErbB3 phosphorylation. These experiments show that c-Src complexes with and mediates phosphorylation of ErbB3. Neither c-Src Tyr kinase activity nor ErbB3 Tyr phosphorylation are, however, necessary for co-immunoprecipitation. This finding has important implications, as discussed below, concerning the underlying mechanism for the interaction between these two Tyr kinases.

Discussion

The ErbB family of receptors produces both proliferative and anti-apoptotic signaling in carcinoma cells, and are targets for therapeutic intervention in the treatment of solid tumors. Breast carcinomas typically express multiple members of the ErbB receptor family, each of which can regulate downstream signal transduction pathways [24]. Our prior observations in squamous and breast carcinoma cells demonstrate that studying a single ErbB receptor cannot fully predict the complexity of ErbB dependent cellular signaling when multiple ErbB receptors are co-expressed. Instead, examination of all ErbB receptors expressed by a given cell identifies concurrent survival signaling resistant to specific inhibition of EGFR or ErbB2 [23]. The complexity of ErbB receptor network signaling is affirmed in the present

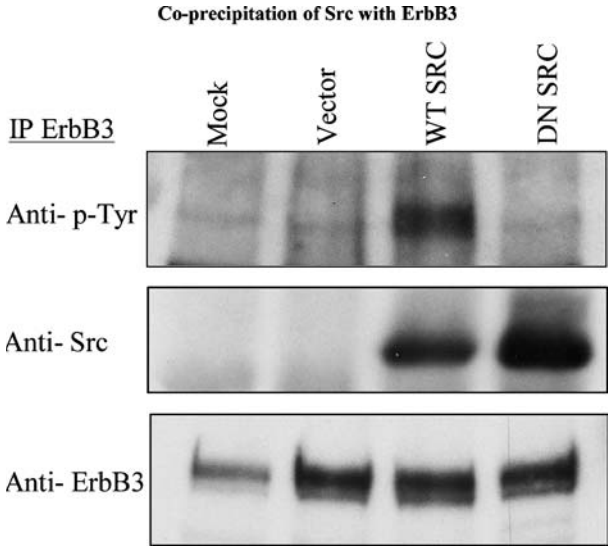


Figure 7. Co-precipitation of ErbB3 and c-Src. A CHO cell line with stable expression of ErbB3 (CHO-ErbB3) was generated. These cells were transiently transfected with either a control vector, a WT c-Src, or a kinase inactive c-Src. Following ErbB3 immunoprecipitation and western blotting, the nitrocellulose membrane was divided and probed for phospho-Tyr or for c-Src. The blot was then stripped and reprobed to insure equal loading of ErbB3.

report through the demonstration of a compensatory ErbB3 signaling response following inhibition of EGFR, ErbB2, and ErbB4.

In this investigation we examined activation of each ErbB receptor family member following over-expression of ErbB cytoplasmic domain deletion mutants and disruption of receptor dimerization. Our results demonstrate distinct inhibitory patterns for each ErbB mutant indicating functional differences for each extracellular domain in dimerization. The different ErbB receptor expression profiles of each breast carcinoma cell line demonstrate potent ErbB receptor network inhibition by the EGFR-CD533 with an important exception. In T47D cells, inhibition of EGFR, ErbB2, and ErbB4 was associated with an enhanced phosphorylation of ErbB3. Blocking this compensatory Tyr kinase activity by pharmacologic c-Src inhibition also inhibited anti-apoptotic Bcl family protein expression. Furthermore we demonstrate that abrogating ErbB receptor and c-Src activity, through EGFR-CD533 expression in MDA-MB231 cells or EGFR-CD533 expression and PP2 treatment in T47D cells, enhances radiation-induced apoptosis. Finally we establish that enhanced expression of ErbB3 also supplies a c-Src dependent anti-apoptotic signal independent of EGFR and/or ErbB2 activities and indeed represents a compensatory response to the inhibition of the EGFR (summarized in Figure 8).

The relative effects of EGFR-CD533 and ErbB2-CD572 illustrate differences in extracellular domain interactions between the two receptors. In MDA-MB231 cells, both deletion mutants are equally inhibitory for EGFR and ErbB2. However in T47D cells, which express a different complement and proportion of ErbB receptors, the ErbB2 deletion mutant does not affect EGF directed EGFR/ErbB2 Tyr phosphorylation. EGFR-CD533 expression, on the other hand, blocks EGF directed signaling in both carcinoma cell lines. One explanation for the potent inhibitory effects of the EGFR-CD533 is a dominance of inhibitory homo-

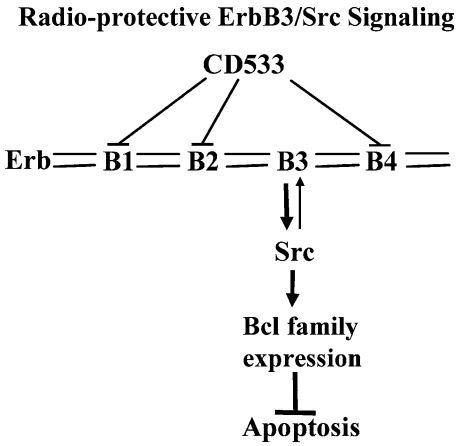


Figure 8. Radioprotective ErbB3/c-Src signaling. The flow diagram illustrates compensatory ErbB3/c-Src/Bcl family anti-apoptotic signaling following inhibition of other co-expressed ErbB RTKs.

dimerizations between WT EGFR and the EGFR-CD533 deletion mutant. This concept is supported by experiments demonstrating a preference for homo-dimerization among EGFR extracellular domains [35]. The observations that EGFR-CD533, but not ErbB2-CD572, blocks EGFR Tyr phosphorylation in cells with low EGFR expression (T47D) suggests that the EGFR-CD533 maintains its ability to preferentially form inhibitory homo-dimeric complexes while the ErbB2-CD572 does not. Additionally we provide further evidence for the broad inhibitory activity of EGFR-CD533 through hetero-dimerization since its expression inhibits even HRG-directed stimulation of ErbB2 and ErbB4.

The ErbB2-CD572 also differs from the EGFR-CD533 by producing graded and incomplete inhibition of ErbB receptor phosphorylation (Figure 2). For example, following HRG β stimulation in T47D cells, ErbB2-CD572 only partially inhibits ErbB2 and ErbB3 activation. This non-specific pattern of ErbB receptor inhibition suggests that unlike the EGFR-CD533, the ErbB2-CD572 mutant does not engage in preferential receptor dimerizations. This lack of specificity explains the deficiency of ErbB2-CD572 in blocking EGFR phosphorylation at low expression levels (e.g. T47D cells). This interpretation is in agreement with current concepts of ErbB2 as a mediator of lateral signaling and a dimerization partner for other ErbB receptors [6,36]. Our results suggest that although the extracellular domain of ErbB2 does not confer specificity for dimerization, it facilitates ErbB2 interaction with other receptors in a flexible manner, depending on the ErbB expression pattern of a given cell.

A novel functional role for the ErbB3 receptor has also been identified in this investigation. ErbB3 is unique amongst the other ErbB receptors because it does not have an active kinase domain [8]. This implies that ErbB3 alone cannot generate phosphorylation dependent, downstream signals. Instead, ErbB3 depends upon dimerization with other ErbB receptors and subsequent trans-phosphorylation to preferentially recruit proteins such as p85/PI3K [37,38] and Grb7 [9] to the plasma membrane. Our data provides evidence that ErbB3 recruits c-Src signaling, similar to EGFR and ErbB2 [26,27], and mediates anti-apoptotic signaling in part through Bcl family protein expression. Although ErbB3 signaling via PI3K/Akt represents an important anti-apoptotic pathway in some cells, we are unable to demonstrate compensatory enhancements in Akt signaling in T47D cells (data not shown).

Our findings parallel studies of kinase defective mutants of the EGFR, which are conceptually similar to the wild type ErbB3. Like ErbB3, kinase inactive EGF receptors produce downstream signals following trans-phosphorylation by other Tyr kinases [39–41]. In one study [40], ectopic expression of a kinase inactive EGFR mutant in a hematopoietic cell line generated cell survival signals similar to those described in this report and were dependent upon c-Src activity. Thus it is possible that kinase-independent signaling of ErbB receptors

through c-Src is a more generalized mechanism common to all ErbB receptor family members.

Our results raise an important question regarding the nature of interactions between ErbB3 and c-Src. According to models of c-Src regulation [42,43], either SH2-phospho-Tyr or SH3-polyproline interactions can alter c-Src protein conformation and enhance its activity. Our experiments with an ectopic expression system demonstrate that physical interactions between ErbB3 and c-Src do not depend on ErbB3 receptor Tyr phosphorylation (Figure 6), and therefore may not involve SH2 interactions. Instead we suggest that COOH-terminal proline-rich sequences or some other determinant in ErbB3 may serve as a docking site for c-Src domains. The molecular basis of this interaction is intriguing and requires further investigation. Studies characterizing the corresponding ErbB3 phosphorylation sites by mass spectrometry are in progress.

The various ErbB receptor expression profiles of different carcinomas and the expanding knowledge about ErbB receptor cellular functions have made this receptor family molecular targets for new strategies in blocking tumor cell growth. While several interventions have shown promise in both pre-clinical and clinical studies, an emerging body of literature has identified either acquired or compensatory resistance mechanisms to specific ErbB receptor inhibition. For example, enhanced vascular-endothelial growth factor expression in xenograft tumors has been demonstrated to provide resistance to monoclonal antibodies directed against the EGFR [44]. Resistance has also been shown *in vitro*, where pharmacologic inhibition of EGFR Tyr kinase activity results in the upregulation of insulin-like growth factor receptor expression and signaling [45]. We have employed genetic manipulation of ErbB receptor signaling to show that ErbB3 and c-Src also provide a compensatory response to ErbB receptor inhibition. This novel signaling pathway protects carcinoma cells from apoptosis, implicating ErbB3 expression and signaling as important factors to consider during therapeutic targeting of ErbB receptors. For breast carcinomas, where ErbB3 is expressed in approximately 50% of invasive ductal carcinomas [46] and strong immunoreactivity has been measured in 22–50% of tumor samples [47,48], this protective response may be particularly relevant. Our data suggests that breast carcinomas expressing low or undetectable levels of ErbB3 are more susceptible to current ErbB receptor inhibition strategies and that combined ErbB and c-Src inhibition may provide a benefit in decreasing breast carcinoma cell survival.

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