A Novel Association between p130Cas and Resistance to the Chemotherapeutic Drug Adriamycin in Human Breast Cancer Cells

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Abstract

Resistance to chemotherapy remains a major obstacle for the treatment of breast cancer. Understanding the molecular mechanism(s) of resistance is crucial for the development of new effective therapies to treat this disease. This study examines the putative role of p130Cas (Cas) in resistance to the cytotoxic agent Adriamycin. High expression of Cas in primary breast tumors is associated with the failure to respond to the antiestrogen tamoxifen and poor prognosis, highlighting the potential clinical importance of this molecule. Here, we show a novel association between Cas and resistance to Adriamycin. We show that Cas overexpression renders MCF-7 breast cancer cells less sensitive to the growth inhibitory and proapoptotic effects of Adriamycin. The catalytic activity of the nonreceptor tyrosine kinase c-Src, but not the epidermal growth factor receptor, is critical for Cas-mediated protection from Adriamycin-induced death. The phosphorylation of Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) is elevated in Cas-overexpressing cells treated with Adriamycin, whereas expression of the proapoptotic protein Bak is decreased. Conversely, Cas depletion increases sensitivity to the death-inducing apoptosis in the presence of Adriamycin. Because Cas is frequently expressed at high levels in breast cancers, these findings raise the possibility of sensitizing Cas-overexpressing tumors to chemotherapy through perturbation of Cas signaling pathways. [Cancer Res 2008;68(21):8796–804]

Introduction

Breast cancer is a major cause of cancer-related death in women, second only to lung cancer.1 Chemotherapy has been shown to benefit most women with breast cancer, especially those patients whose tumors are estrogen receptor (ER)−negative or who have become refractory to hormone therapy (1). In the United States, one of the most frequently used agents for treating breast cancer is the chemotherapeutic drug Adriamycin (doxorubicin). When Adriamycin is given as a single-agent treatment, response rates are typically 40% to 60% and can be as high as 80% (2). You, despite its efficacy against breast cancer, resistance to Adriamycin is a major clinical problem and an important cause for treatment failure. Several mechanisms have been suggested to cause resistance to Adriamycin in breast tumor cells. They include the overexpression of P-glycoprotein and other plasma membrane multidrug transporters (2, 3); failure to undergo apoptosis caused by alterations in Bcl-2, Bcl-XL, or Bax expression (4–6); and alterations in drug targets, such as topoisomerase II (7). However, because treatments targeting these pathways have been met with little success, additional factors must also play a role in promoting resistance to Adriamycin. Identification of these mechanisms will facilitate the development of more effective strategies to overcome Adriamycin resistance in breast cancer.

A number of cytoplasmic signaling molecules have been implicated in resistance of breast tumor cells to Adriamycin, including focal adhesion kinase, Akt, and extracellular signal-regulated kinase 1/2 (ERK1/2; refs. 8, 9). Expression of the scaffolding molecule p130Cas (Cas; also known as breast cancer antigen 1 [BCAR1]) was found to be up-regulated in MCF-7 and ZR-75-1 breast cancer cells treated with Adriamycin (10). Interestingly, high expression of Cas in primary breast tumors correlates with the failure to respond to the antiestrogen tamoxifen and poor prognosis (11). Cas overexpression in estrogen-dependent ZR-75-1, T47D, and MCF-7 breast cancer cells is sufficient to drive proliferation in the presence of the antitamoxifen and ICI 182,780 (12, 13). Despite the fact that these cells proliferate in the presence of tamoxifen under conditions of high Cas expression, gene expression driven from estrogen-regulated promoters is generally inhibited (12, 13). This suggests that Cas overexpression activates cell proliferation pathways that are independent of ER-dependent gene regulation. Work from our group has shown that these pathways include the protein tyrosine kinase c-Src, signaling from the epidermal growth factor receptor (EGFR) and signal transducer and activator of transcription 5b (STAT5b; ref. 13).

In this study, we show that, in addition to tamoxifen resistance, Cas mediates resistance to the apoptotic and antiproliferative effects of Adriamycin in human breast cancer cells. Cas-mediated protection from Adriamycin requires the kinase activity of c-Src, but not that of the EGFR. Cas overexpression promotes activation of Akt and ERK1/2 in the presence of Adriamycin and alters the balance of Bcl-2 family members in favor of the antiapoptotic proteins. Conversely, Cas depletion increases sensitivity to the death-inducing effects of Adriamycin. Based on these findings, we suggest that Adriamycin resistance, mediated by Cas, results from the sum of these mechanisms will facilitate the development of more effective strategies to overcome Adriamycin resistance in breast cancer.

Materials and Methods

Cell culture. Stable tetracycline-regulated MCF-7 clones, containing either pTrev2-Pur (Vector) or Myc-Cas-pTrev2-Pur (Cas4), were previously

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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1 www.breastcancer.org
described (13). Clones were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 4 mM/L l-glutamine, 100 μg/mL G418, and 0.75 μg/mL puromycin. T47D cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 1× glucose, 10 mM/L HEPEs, 1 mM/L sodium pyruvate, and 7 μg/mL insulin. MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1 mM/L sodium pyruvate.

Reagents and immunoblotting. Western blots were performed, as described previously (14, 15). Antibodies obtained were as follows: phosphorylated Akt (S473), total Akt, Bcl-2, Cas, Crk, and poly(ADP-ribose) polymerase 1 (PARP; BD Biosciences); phosphorylated ERK1/2, actin, and β-tubulin (Sigma); ERK (Cell Signaling Technology); Bak (Upstate); Bax, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology); and Bcl-XL (Millipore). Doxycycline, Adriamycin, and RNase (Sigma); PP2 and AG1478 (Calbiochem); rhodamine 123 (R123) and propidium iodide (PI; Molecular Probes); and Dnase I (Invitrogen) were purchased from the indicated sources.

R123 incorporation. Cells (2.5 × 10^5) were plated in 60-mm dishes and cultured in the presence or absence of 1 μg/mL doxycycline for 48 h. Cells were washed and placed in medium supplemented with vehicle or the indicated concentration of Adriamycin in the presence or absence of 1 μg/mL doxycycline. The cells were collected 48 h later, incubated with the membrane-permeable lipophilic cationic fluorochrome R123 (200 mM/L), and analyzed by flow cytometry.

RNA interference and transfections. BCAR1 stealth select RNA interference (siRNA) RNA interference (siRNA; siControl) were purchased from Invitrogen and Dharmacon Research, respectively. A second oligonucleotide (GUCUACGACGUUCCUCAU) was synthesized directed against the YXXP substrate-binding domain of Cas (siXXXP). Transfections were conducted in six-well plates using Lipofectamine RNAiMax transfection reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, RNA duplexes (600 pmol) were complexed with Lipofectamine RNAiMax in six-well plates for 10 min at room temperature, and 8 × 10^5 cells were subsequently added. At 24 h later, cells were collected, counted, and replated at a concentration of 2.5 × 10^5 cells per condition. Cells were treated 24 h later with the indicated concentrations of Adriamycin, and R123 analysis was performed 48 h later.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and cell cycle determination. Cells (2.5 × 10^5) were plated in 60-mm dishes and cultured in the presence or absence of 1 μg/mL doxycycline for 48 h. Cells were washed and placed in fresh medium supplemented with vehicle or 3 μM/L Adriamycin in the presence or absence of 1 μg/mL doxycycline. Cells were processed 48 h later for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using an in situ cell death detection kit (Roche Applied Science) and analyzed by flow cytometry, or for cell cycle analysis by staining with 40 μg/mL PI solution in the presence of 100 μg/mL RNase followed by flow cytometry. Cell cycle was quantified using the Dem-Jett-Fox modeling algorithm.

Flow cytometry. Adherent cells were trypsinized, collected, combined with floating cells, and fixed using the BD Cytofix/Cytoperm solution (BD Bioscience) for 20 min at 4°C (cells for the R123 studies were not fixed). Cells were processed, as described above, and analyzed on the FACScaliber (Becton Dickinson). Analysis of data was performed using the Flowjo flow cytometry software (Tree Star, Inc.).

Densitometry. Densitometry was performed by the Molecular Dynamics densitometer (Molecular Dynamics) and quantified with ImageQuant 5.0 software (GE).

Statistical analyses. Two-tailed Student’s t tests were used for the pairwise comparison of experimental groups.

Results

Differential sensitivity of human breast cancer cell lines to Adriamycin. One mechanism by which Adriamycin exerts its cytotoxic activity is through induction of apoptosis via the mitochondrial intrinsic pathway (16). To begin to investigate the role of Cas in Adriamycin resistance, we determined the sensitivity of several human breast cancer cell lines to Adriamycin using R123 incorporation, a measure of mitochondrial transmembrane potential. MCF-7, T47D, and MDA-MB-231 (231) cells were treated with increasing concentrations of Adriamycin for 48 hours, and R123 incorporation was analyzed by flow cytometry. T47D cells (ER+, model of early-stage breast cancer) were completely insensitive to the death-inducing effects of Adriamycin (Fig. 1A, squares), whereas MDA-MB-231 cells (ER−, model of more advanced stage breast cancer) showed a 30% decrease in R123 incorporation at the maximum drug concentration (diamonds). MCF-7 cells (ER+, model of early-stage breast cancer) were the most sensitive cell line, displaying an initial 50% reduction in R123 incorporation at 1 μM/L Adriamycin and an additional 10% decline at 3 to 20 μM/L (triangles). These results show that sensitivity to Adriamycin differs among human breast cancer cell lines and neither ER status nor tumor cell “aggressiveness” seems to be reliable predictors of sensitivity to this drug.

In a previous study, we noted that Cas expression was variable in human breast cancer cell lines (17). To test whether the differing sensitivity to Adriamycin could be due to Cas levels in the cell, Cas expression was measured in the three cell lines. Cas was expressed in the resistant T47D cell line at levels that were 2.1-fold above those seen in the sensitive MCF-7 cell line (Fig. 1B). MDA-MB-231 cells, which exhibited intermediate sensitivity to Adriamycin, contained 1.7-fold greater amounts of Cas relative to MCF-7 cells. Interestingly, the Cas that was present in T47D and MDA-MB-231 contained 1.7-fold greater amounts of Cas relative to MCF-7 cells. MCF-7, T47D, and MDA-MB-231 were washed and placed in medium supplemented with vehicle or the indicated concentration of Adriamycin in the presence or absence of 1 μg/mL doxycycline for 48 hours, and R123 incorporation was analyzed by flow cytometry. T47D cells (ER+, model of early-stage breast cancer) were completely insensitive to the death-inducing effects of Adriamycin (Fig. 1A, squares), whereas MDA-MB-231 cells (ER−, model of more advanced stage breast cancer) showed a 30% decrease in R123 incorporation at the maximum drug concentration (diamonds). MCF-7 cells (ER+, model of early-stage breast cancer) were the most sensitive cell line, displaying an initial 50% reduction in R123 incorporation at 1 μM/L Adriamycin and an additional 10% decline at 3 to 20 μM/L (triangles). These results show that sensitivity to Adriamycin differs among human breast cancer cell lines and neither ER status nor tumor cell “aggressiveness” seems to be reliable predictors of sensitivity to this drug.

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Figure 1. Human breast cancer cell lines differ in their sensitivity to Adriamycin. A, 2 × 10^5 MCF-7, T47D, and MDA-MB-231 cells were plated in 60-mm dishes and allowed to adhere for 24 h. Cells were treated with 0 to 20 μM/L Adriamycin for an additional 48 h, and the mitochondrial transmembrane potential was measured by R123 incorporation and flow cytometry, as described in Materials and Methods. Points, mean of three independent experiments; bars, SE. B, expression of Cas in human breast cancer cell lines. Proteins (50 μg) were isolated from MCF-7, T47D, and MDA-MB-231 cells and immunoblotted for Cas and the small adaptor molecule Crk, as a loading control. Densitometric values represent the ratio of Cas to loading control (Crk) relative to the value obtained for MCF-7 cells.
cells displayed retarded electrophoretic mobility compared with Cas in MCF-7 cells; this slower migrating species represents the hyperphosphorylated form of Cas (18). Phosphorylation of Cas, which is predominately mediated by c-Src (19), plays a critical role in its function as a regulator of survival and proliferation (14, 20, 21). Thus, the elevated expression of highly phosphorylated Cas in resistant cells suggests that Cas may play a role in the cellular response to Adriamycin through its ability to modulate growth and survival pathways.

**Cas promotes resistance to Adriamycin.** To address whether Cas expression has an effect on Adriamycin-induced cell death, MCF-7 breast cancer cells that stably express tetracycline-regulated (Tet-off) constructs encoding vector sequences or Myc-Cas were grown in the presence or absence of 1 μg/mL doxycycline for 48 h to modulate Cas levels. Cas was expressed ~6-fold over endogenous levels in the absence of doxycycline (Fig. 2A, inset). These cells were then cultured for an additional 48 h in 0 to 20 μm/L Adriamycin in the presence or absence of doxycycline, and R123 incorporation was assessed by flow cytometry. In the presence of increasing concentrations of Adriamycin, the percentage of cells incorporating R123 was consistently reduced by ~40% in cells expressing endogenous (1×) Cas compared with cells with 6-fold above endogenous levels of Cas (Fig. 2A). These differences in mitochondrial transmembrane potential suggest that Cas may promote resistance to Adriamycin through up-regulation of survival signals emanating from the mitochondria.

To further evaluate the effect of Cas overexpression on Adriamycin-induced apoptosis, TUNEL staining was performed on Cas-inducible or vector-controlled MCF-7 cells cultured, as described above. Adriamycin treatment induced apoptosis, as measured by TUNEL positivity, in vector-control cells and in the Cas-inducible cell line when Cas was expressed at endogenous levels (Fig. 2B, gray and black columns). Cas overexpression significantly inhibited Adriamycin-induced apoptosis (checkered-black column).

In parallel, the effect of Adriamycin on the cell cycle was assessed as a function of Cas expression by determining the percentage of cells in G0-G1, S, and G2-M. Cells were cultured as described above, stained with PI, and analyzed by flow cytometry to determine the percentage of cells in each phase (Supplementary Fig. S1). Cas overexpression induced a significant increase in the percentage of cells in the S phase regardless of treatment (Fig. 2C). Adriamycin caused a significant increase in G0-G1 and a concomitant decrease in the percentage of cells in G1. Cell cycle was measured by R123 incorporation and flow cytometry, as described in Materials and Methods. *Points, mean of three independent experiments; bars, SE. **Significant difference from the mean at >95% confidence interval (95% CI) relative to cells expressing endogenous levels of Cas under the same treatment conditions. Inset, Cas4 cells were cultured in the presence or absence of 1 μg/mL doxycycline for 48 h. Proteins were isolated from these cells and immunoblotted for Cas and GAPDH.

**Figure 2.** Cas promotes resistance to Adriamycin. A, cells overexpressing Cas incorporate greater amounts of R123. Stable doxycycline-regulated MCF-7 (Cas4) cells were cultured in the presence (diamonds) or absence (squares) of 1 μg/mL doxycycline (Dox) for 48 h and then treated with 0 to 20 μm/L Adriamycin for an additional 48 h in the presence or absence of doxycycline. Mitochondrial transmembrane potential was measured by R123 incorporation and flow cytometry, as described in Materials and Methods. Points, mean of three independent experiments; bars, SE. *Significant difference from the mean at >95% confidence interval (95% CI) relative to cells expressing endogenous levels of Cas under the same treatment conditions. Inset, Cas4 cells were cultured in the presence or absence of 1 μg/mL doxycycline for 48 h. Proteins were isolated from these cells and immunoblotted for Cas and GAPDH. B, Cas overexpression renders cells resistant to the apoptosis-inducing effect of Adriamycin. Cells were cultured as described in A. Apoptosis was measured by TUNEL positivity and flow cytometry, as described in Materials and Methods. Columns, mean of four independent experiments; bars, SE. **Significant difference from the mean at >95% confidence interval (95% CI) relative to cells expressing endogenous levels of Cas under the same treatment conditions. C, Cas overexpression abrogates Adriamycin-induced G0-G1 arrest and results in a greater percentage of cells in S phase. Cells were cultured as described in A. Cell cycle was measured by PI staining and flow cytometry, as described in Materials and Methods. The data are the mean of three independent experiments. D, Cas depletion sensitizes cells to Adriamycin. MDA-MB-231 or T47D cells were transfected with mock, control, or Cas-targeted siRNAs and then treated with 0 to 2 μm/L Adriamycin for 48 h. Mitochondrial transmembrane potential was measured by R123 incorporation and flow cytometry, as described in Materials and Methods. Columns, mean of three independent experiments; bars, SE. **Significant difference from the mean at >95% confidence interval (95% CI) relative to mock-transfected cells under the same treatment conditions. In parallel, proteins were isolated from siRNA-treated cells and immunoblotted for Cas and GAPDH.
in the percentage of cells in the S phase when Cas was expressed at endogenous levels, indicative of a G0-G1 arrest. In contrast, cells overexpressing Cas exhibited no G0-G1 block, and as stated above, the percentage of cells in S phase increased.

We hypothesized that, if Cas overexpression could render sensitive cells resistant to Adriamycin, then depletion of Cas in resistant cells might have the opposite effect. T47D cells that exhibited the greatest level of resistance to Adriamycin, were depleted for Cas with the use of targeted siRNAs (Fig. 2D, top left) and cultured in the presence of 0 to 2 μmol/L Adriamycin for 48 hours. As expected, treatment of these cells with Adriamycin alone had no significant effect on R123 incorporation (Fig. 2D, left graph, black columns). However, cells treated with Cas-targeted siRNAs showed a significant reduction in R123 incorporation after Adriamycin treatment (siCas, gray columns) compared with mock-transfected and siControl-treated cells (black and white columns, respectively). Thus, upon Cas depletion, T47D cells become sensitized to Adriamycin-induced apoptosis.

To examine whether this was unique to the T47D cell line, we also investigated the effect of Cas depletion in MDA-MB-231 cells, which showed an intermediate sensitivity to Adriamycin. Again, Adriamycin had no significant effect on mock-transfected or siControl-treated MDA-MB-231 cells (Fig. 2D, right graph, black and white columns). However, Cas depletion by siCas resulted in a significant decrease in R123 incorporation in the presence of 2 μmol/L Adriamycin (gray column). To exclude nonspecific effects, similar Cas depletion experiments were performed in MDA-MB-231 cells using a second independent siRNA oligonucleotide targeting Cas (siYXXP). As was the case for siCas, MDA-MB-231 cells treated with siYXXP exhibited greater sensitivity to Adriamycin than did control cells (Supplementary Fig. S2). Taken together, these results indicate that Cas depletion reverses chemoresistance and sensitizes these cells to the cytotoxic effects of Adriamycin.

**Cas-dependent Adriamycin resistance requires the kinase activity of c-Src.** We and others have previously shown that Cas is a potent activator of c-Src kinase activity through its ability to bind to the Src homology 2 (SH2) and SH3 domains, thus relieving its autoinhibitory conformation (14, 20, 22). To test the requirement for c-Src kinase activity in Cas-mediated resistance to Adriamycin, Cas-inducible MCF-7 cells were treated with siYXXP and cultured in the presence of 0 to 2 μmol/L Adriamycin and then analyzed for R123 incorporation. Consistent with the data in Fig. 2A, Adriamycin treatment resulted in a marked decrease in R123 incorporation in cells expressing endogenous Cas, whereas cells overexpressing Cas showed significantly greater R123 incorporation (Fig. 3A; medium, Adriamycin-treated cells). However, in the presence of PP2, Cas overexpression was no longer seen to protect the cells from the toxic effects of Adriamycin (compare white columns in Adriamycin-treated medium versus PP2 groupings).

TUNEL assays were performed to confirm these results. As was the case in Fig. 2B, the extent of apoptosis induced by Adriamycin was significantly reduced in cells overexpressing Cas (Fig. 3B; medium, Adriamycin-treated cells). PP2 treatment resulted in a significant increase in apoptosis under both control and Cas-overexpressing conditions when cells were cultured in Adriamycin (PP2, Adriamycin-treated cells). In addition, the protective effect of Cas overexpression on Adriamycin-induced cell death was significantly less in the presence of PP2. Whereas Cas overexpression resulted in ~70% decrease in the number of TUNEL-positive cells when cultured in the presence of Adriamycin (Fig. 3C; medium, Adriamycin-treated cells), only ~32% decrease was observed in the presence of both Adriamycin and PP2 (PP2, Adriamycin-treated cells). Interestingly, Cas overexpression also had a protective effect on the low level of TUNEL positivity observed in the absence of Adriamycin, resulting in ~80% decrease in the number of TUNEL-positive cells (medium, vehicle-treated cells). This effect was similar in PP2-treated cells in the absence of

![Figure 3](image-url)
Adriamycin (PP2, vehicle-treated cells). These data indicate that c-Src kinase activity contributes specifically to Cas-dependent protection from Adriamycin-induced apoptosis.

Because EGFR signaling contributes to Cas-mediated antiestrogen resistance (13), we next investigated whether EGFR catalytic activity was required for Cas-dependent resistance to Adriamycin. Cas-overexpressing cells grown in the presence or absence of 1 μg/mL doxycycline were treated with 3 μmol/L Adriamycin and the EGFR kinase inhibitor AG1478. Cas overexpression continued to provide protection from the toxic actions of Adriamycin in the presence of AG1478, as seen by R123 incorporation (Fig. 3A). Cas overexpression resulted in a 72% decrease in the number of TUNEL-positive cells cultured in the presence of both Adriamycin and AG1478 (Fig. 3C; AG1478, Adriamycin-treated cells), similar to the ~70% reduction in the presence of either agent alone. Thus, in contrast to c-Src activity, EGFR kinase activity did not seem to contribute to the protective effect of Cas overexpression against the cytotoxic activity of Adriamycin.

Cas overexpression is associated with the activation of Akt and ERK1/2. Because Cas expression seemed to play a role in the cellular response to Adriamycin, we next investigated whether drug treatment affected the level of Cas in the cell. Interestingly, endogenous Cas was markedly reduced after Adriamycin treatment (Fig. 4A, top, compare lanes 1 and 3 and B, left), but the level of Cas remained high in cells overexpressing Cas (Fig. 4A, lane 4 and B). The phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which can be activated in response to both c-Src activation and Cas overexpression (23), contributes to chemoresistance and radioresistance (24). To assess whether Cas overexpression affects Akt activity, phosphorylation of S473 on Akt was evaluated. Akt phosphorylation was not altered by Cas expression in the absence of drug (Fig. 4A, second panel and B, middle). However, pAkt S473 became significantly elevated in Cas-overexpressing cells after 48 hours of Adriamycin treatment.

ERK1/2 has also been reported to be activated in response to Cas overexpression and c-Src activation (22, 23). Phosphorylated ERK1/2 levels were measured to determine whether ERK1/2 is differentially activated during Adriamycin treatment in the presence of high levels of Cas. ERK1/2 phosphorylation was elevated in Cas-overexpressing cells compared with cells expressing endogenous levels of Cas, irrespective of whether the cells were treated with Adriamycin or not (Fig. 4A, fourth panel, lanes 2 and 4 and B, right, white columns). Whereas total ERK1/2 levels decreased in the presence of Adriamycin, phosphorylated ERK1/2 was selectively enhanced in cells expressing either endogenous or overexpressed levels of Cas. However, phosphorylated ERK1/2 was elevated to a greater extent in Cas-overexpressing cells compared with cells expressing endogenous Cas.

Cas regulates the expression of Bcl-2 family members. The changes in R123 incorporation reported above suggest that Cas protects against mitochondrial membrane damage after Adriamycin treatment. We hypothesized that Cas expression might affect the expression of one or more of the Bcl-2 family members that control the intrinsic death pathway. To test this hypothesis, the expression of Bcl-2 family members was measured in MCF-7 cells induced to overexpress Cas. Expression of the proapoptotic protein Bak was decreased by ~40% as a function of Cas overexpression (Fig. 5A, second panel, compare lanes 1 and 2, and densitometry). Adriamycin treatment had little effect on Bak expression in cells expressing endogenous Cas (lane 3). However, Bak expression was significantly reduced when cells overexpressing Cas were treated with Adriamycin (lane 4). This result was consistently observed in four independent experiments. In contrast to Bak, expression of the proapoptotic protein Bax and the antiapoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> did not change in response to Adriamycin treatment or Cas expression levels (third to fifth panels).

The corresponding loss-of-function approach was taken in MDA-MB-231 cells to assess whether depletion of Cas in Adriamycin-resistant cell lines would also affect the expression of Bcl-2 family members. Cas depletion resulted in increased levels of Bak (2.6-fold) relative to control cells in the absence of Adriamycin (Fig. 5B, second panel and densitometry), demonstrating that Bak expression can be regulated by Cas in these cells under normal growth conditions. However, when the cells were treated with Adriamycin, siControl-treated and siCas-treated cells contained

![Figure 4](https://cancerres.aacrjournals.org/toc/content/68/13/8800/DC1/27326240464.png)

**Figure 4.** Cas overexpression correlates with increased activation of Akt and ERK1/2. **A**, proteins (50 μg) isolated from cells cultured in the presence or absence of Adriamycin for 48 h were separated on 10% SDS-PAGE and immunoblotted with the indicated antibodies. Immunoblots shown are representative of three experiments. **B**, relative protein expression levels. The relative expression levels of Cas, pAkt S473, and pERK1/2 were determined by dividing the band intensities obtained by densitometry for the proteins of interest by the band intensities of GAPDH (Cas), Akt (pAkt S473), or ERK1/2 (pERK1/2). These values were then normalized to the corresponding value for vehicle-treated cells expressing 1 X Cas. Columns, mean of three independent experiments; bars, SE. *, significant difference from the mean at >95% CI.
roughly equivalent levels of Bak, as determined by densitometry. Bax and Bcl-2 expression did not change significantly in MDA-MB-231 cells in response to Cas depletion or Adriamycin treatment (third and fourth panels). However, expression of the antiapoptotic protein Bcl-XL was completely abrogated under conditions of Cas depletion or Adriamycin treatment (fifth panel, lanes 2–4). Similar results were seen in T47D cells (data not shown). This change effectively creates an imbalance in Bcl-2 proteins in favor of the proapoptotic members. Interestingly, Adriamycin did not completely abrogate endogenous Cas expression in siControl-treated MDA-MB-231 cells as it did in MCF-7 cells (top, lane 3). Moreover, only the slower migrating, hyperphosphorylated form of Cas remained. We suggest that the prosurvival signals resulting from the continued presence of phosphorylated Cas may outweigh proapoptotic signals generated by this shift in the balance of Bcl-2 proteins when control cells are treated with Adriamycin.

Figure 5. Cas mediates resistance to Adriamycin through modulation of the mitochondrial-mediated cell death pathway. Proteins (50 μg) isolated from cells cultured in the presence or absence of Adriamycin for 48 h were separated by SDS-PAGE and immunoblotted with the indicated antibodies. The relative expression levels of Cas and Bak were determined by dividing the band intensities obtained by densitometry for the proteins of interest by the band intensities of the loading control (β-tubulin or GAPDH). These values were then normalized to the corresponding value for vehicle-treated cells expressing 1X Cas (A) or vehicle-treated cells transfected with control siRNAs (B). Columns, mean of three (A) and two (B) independent experiments; bars, SE. *, significant difference from the mean at ≥95% CI. Immunoblots in C are representative of three independent experiments.
Adriamycin-induced cell death also depends on activation of caspases, which play crucial roles in the proteolysis of a number of key targets (25). Thus, we hypothesized that Cas overexpression might result in the inhibition of caspase activation. One target of caspase activity is Cas, which has been reported to be cleaved during etoposide-induced apoptosis (26). As discussed above, endogenous Cas was significantly diminished in response to Adriamycin treatment in both MCF-7 and MDA-MB-231 cells. This coincided with the appearance of a 31-kDa cleavage product after Adriamycin treatment (Supplementary Fig. S3). Both the decrease in full-length Cas and the accumulation of the 31-kDa fragment were less pronounced in the presence of the caspase inhibitor z-VAD-fmk (data not shown), implicating caspases in the Adriamycin-induced proteolysis of endogenous Cas. Interestingly, the 31-kDa fragment was also present in Adriamycin-treated Cas-overexpressing cells (Supplementary Fig. S3). However, full-length Cas is still expressed at high levels under these conditions (Figs. 4A and 5A). To examine caspase activity at the molecular level, the extent of cleavage of the caspase substrate PARP1 was measured in the Cas-inducible MCF-7 cells. In the absence of Adriamycin, PARP1 was present as a full-length, 118-kDa species in both control and Cas-overexpressing cells (Fig. 5C, lanes 1 and 2). After Adriamycin treatment, PARP1 was cleaved in control cells, resulting in a reduction in the 118-kDa species and the appearance of an 85-kDa fragment (lane 3). Cleavage of PARP1 was significantly reduced in Cas-overexpressing cells after Adriamycin treatment (lane 4), suggesting that overexpression of Cas blocked the activation of the caspases responsible for PARP1 cleavage.

Discussion

Cas is overexpressed in a large number of breast cancers, coincident with increased resistance to tamoxifen and poor relapse-free and overall survival rates (11). Here, we have examined the putative role of Cas in resistance to the cytotoxic agent Adriamycin. We show for the first time that MCF-7 cells overexpressing Cas are less sensitive to the growth inhibitory and proapoptotic effects of Adriamycin. The kinase activity of c-Src, but not that of the EGFR, is required for Cas-mediated enhancement of cell proliferation and survival in the presence of Adriamycin. Akt and ERK1/2 activation are up-regulated when cells overexpressing Cas are treated with Adriamycin, coincident with a down-regulation in Bak expression. This results in a shift toward prosurvival/proliferation signals stemming from Akt and ERK1/2 and away from proapoptotic Bcl-2 proteins. Based on these findings, we propose that Cas overexpression in sensitive cells activates growth and survival pathways that are regulated by c-Src, Akt, and ERK1/2 and leads to the inhibition of mitochondrial-mediated cell death and promotion of continued proliferation in the presence of Adriamycin (Fig. 6A). Conversely, Cas depletion in more resistant cell lines results in a shift toward proapoptotic Bcl-2 proteins, coincident with decreased R123 incorporation in the presence of Adriamycin (Fig. 6B). However, residual expression of hyperphosphorylated Cas, which is seen when siControl-treated cells are treated with Adriamycin, may protect cells from the cytotoxic effects of Adriamycin in the presence of a similar shift toward proapoptotic Bcl-2 signals (Fig. 6C). These results bring to light a novel association between Cas overexpression and Adriamycin resistance, a finding that is relevant to human breast cancer because Cas is often found to be expressed at high levels in breast tumors (11).

Figure 6. Models for Cas-mediated resistance to Adriamycin. A, effect of Cas overexpression in “sensitive” cell lines treated with Adriamycin (MCF-7 cells expressing 6× Cas). B, effect of Cas depletion in “resistant” cell lines treated with Adriamycin (MDA-MB-231 cells treated with siCas). C, effect of “resistant” cells treated with Adriamycin (MDA-MB-231 cells treated with siControl).
showed that Cas overexpression in T47D cells caused increased c-Src and ERK1/2 activities in response to estrogen (22). More recently, our group showed that Cas-dependent resistance to tamoxifen involved a signaling axis that included c-Src, EGFR, and STAT5b (13). Interestingly, whereas the data presented above indicate that c-Src kinase activity is important for Cas-dependent Adriamycin resistance, the catalytic activity of the EGFR seems to be less important. These data are not necessarily inconsistent with our previous data on tamoxifen resistance, because the earlier study implicated EGFR functions that could have been independent of its catalytic activity. It would seem, from the sum of the data, that high Cas expression in breast cancer cells activates potent proliferation/survival programs that override a variety of inputs that would otherwise induce cell cycle arrest and/or death.

One component of the Cas signaling axis that seems to control resistance to Adriamycin is Akt. Others have found a correlation between transient Akt activation and chemoresistance of human breast tumors (8, 27). Cas binds to the p85 subunit of PI3K under a number of conditions, providing a potential link between Cas overexpression and Akt activation (21, 28, 29). In addition to Akt, ERK1/2 activity was found to be up-regulated in Cas-overexpressing cells. Evidence for an association between ERK1/2 activation and chemotherapeutic resistance is somewhat conflicting. Two groups have shown that ERK1/2 activation correlates with protection from cytotoxic drugs (30, 31). However, others report that ERK activation facilitates DNA damage–induced apoptosis (32–34). It would seem from these conflicting data that the role played by ERK1/2 in therapeutic resistance can vary, depending on both the nature of the cellular insult and the cell type.

The data presented above are the first to show a link between Cas overexpression and protection from mitochondrial-mediated cell death. Results from the R123 incorporation studies suggest that Cas overexpression contributes to the maintenance of mitochondrial membrane integrity in the presence of Adriamycin. The proapoptotic Bcl-2 family members, Bak and Bax, are considered to be the gatekeepers of this cell death pathway. In cells expressing endogenous Cas levels, Bak expression remained unchanged 48 hours post–Adriamycin treatment (Fig. 5A). In contrast, Bak expression was reduced when Cas was overexpressed and significantly more decreased when the cells were treated with Adriamycin. Because other Bcl-2 family members were unaffected by Cas levels in the MCF-7 model, this reduction in Bak expression would result in a decrease in the ratio of proapoptotic to antiapoptotic Bcl-2 family members, ultimately leading to reduced activation of the mitochondrial death pathway after Adriamycin treatment. Because Akt and/or ERK1/2 activity have been shown to influence the intrinsic cell death pathway through the regulation of Bcl-2 family proteins (35–38), we propose that Cas overexpression may inhibit mitochondrial-mediated cell death by maintaining a low level of the proapoptotic protein Bak through the combined activation of Akt and ERK1/2. Results from the MDA-MB-231 and T47D models suggest that additional factors may also play a role. Specifically, it seems that expression of hyperphosphorylated Cas, which has been shown to regulate cell growth and survival pathways (14, 20, 21), may override proapoptotic signals stemming from Bcl-2 family members.

Overexpression of human enhancer of filamentation 1 (HEF1), which belongs to the Cas family of adaptor proteins, results in the activation of caspases, cleavage of HEF1, and apoptosis of MCF-7 cells in the absence of any exogenous stress (39). It is noteworthy to mention that the caspase activity in this instance is likely due to caspase-7 or one of the other redundant caspases active in MCF-7 cells, as these cells have been reported to be deficient of caspase-3 (40). Based on the data reported herein, it would seem that Cas and HEF1 function quite differently in controlling cell survival. Rather than inducing cell death, Cas overexpression had positive effects on cell proliferation and survival, both in the presence and absence of Adriamycin (Fig. 2B and C). Coincidentally, Cas overexpression did not result in PARP cleavage, suggesting that caspase activity was not induced under these conditions (Fig. 5C). Nonetheless, Cas has been previously identified as a substrate for caspases during treatment with etoposide, staurosporine, and cisplatin (26, 41). Indeed, we have observed a decrease in full-length Cas after treatment of cells with Adriamycin, and this coincided with the appearance of a 31-kDa carboxyterminal fragment that has been reported to accumulate in response to caspase cleavage of Cas and induce apoptosis in MCF-7 cells (26, 39). Interestingly, the 31-kDa species was detected in Cas-overexpressing cells and may contribute to the low level of apoptosis exhibited by these cells in the presence of Adriamycin (see Fig. 2B). However, full-length Cas remains high under these conditions, coincident with the cells exhibiting greater resistance to the cytotoxic effects of Adriamycin. This suggests that the progrowth and survival activities of full-length Cas may play a dominant role over the apoptotic activities of the 31-kDa fragment in the presence of Adriamycin.

Together, these data support a model whereby the cellular response to cytotoxic therapies, such as Adriamycin, is governed by the balance and integration of proliferation, survival, and death pathways. Because Cas overexpression in human breast tumors is associated with poor prognosis, this study identifies the Cas signaling axis as a potential target that can be exploited to enhance the efficacy of Adriamycin treatment and/or prevent resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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