Protein Kinase A Activation Confers Resistance to Trastuzumab in Human Breast Cancer Cell Lines
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Abstract Purpose: Trastuzumab is a monoclonal antibody targeted to the Her2 receptor and approved for treatment of Her2-positive breast cancer. Among patients who initially respond to trastuzumab therapy, resistance typically arises within 1 year. BT/HerR cells are trastuzumab-resistant variants of Her2-positive BT474 breast cancer cells. The salient feature of BT/HerR cells is failure to downregulate phosphoinositide 3-kinase/Akt signaling on trastuzumab binding. The current work addresses the mechanism of sustained signaling in BT/HerR cells, focusing on the protein kinase A (PKA) pathway.

Experimental Design: We performed microarray analysis on BT/HerR and BT474 cell lines to identify genes that were upregulated or downregulated in trastuzumab-resistant cells. Specific genes in the PKA pathway were quantified using reverse transcription-PCR and Western hybridization. Small interfering RNA transfection was used to determine the effects of gene knockdown on cellular response to trastuzumab. Electrophoretic mobility shift assays were used to measure cyclic AMP-responsive element binding activity under defined conditions. Immunohistochemistry was used to analyze protein expression in clinical samples.

Results: BT/HerR cells had elevated PKA signaling activity and several genes in the PKA regulatory network had altered expression in these cells. Downregulation of one such gene, the PKA-R1α regulatory subunit, conferred partial trastuzumab resistance in Her2-positive BT474 and SK-Br-3 cell lines. Forskolin activation of PKA also produced significant protection against trastuzumab-mediated Akt dephosphorylation. In patient samples, PKA signaling appeared to be enhanced in residual disease remaining after trastuzumab-containing neoadjuvant therapy.

Conclusions: Activation of PKA signaling may be one mechanism contributing to trastuzumab resistance in Her2-positive breast cancer. We propose a molecular model by which PKA confers its effects. (Clin Cancer Res 2009;15(23):7196–206)

The Her2 (erbB2/neu) oncogene encodes a 185-kDa type I receptor tyrosine kinase that belongs to the epidermal growth factor receptor (EGFR) family (reviewed in ref. 1). It is overexpressed in 20% to 25% of invasive breast cancers and its levels correlate strongly with prognosis, thus making it an important therapeutic target in breast cancer (1–4). Trastuzumab (Herceptin; Genentech), approved for the treatment of Her2-positive metastatic breast cancer, is a humanized monoclonal antibody that recognizes a juxtamembrane epitope in the extracellular domain of Her2 (5). Trastuzumab inhibits the growth of Her2-dependent breast cancer cell lines in culture (6–8) and in xenograft animal models (9, 10). The mechanisms by which trastuzumab inhibits growth of Her2-overexpressing cancer cells are not completely defined, but downmodulation of phosphoinositide 3-kinase (PI3K)/Akt and/or Ras/mitogen-activated protein kinase signaling pathways are essential features of trastuzumab response leading to eventual cell cycle arrest (11).

Clinical data show that patient response rates to trastuzumab range from 12% to 34% when it is used as monotherapy in the metastatic setting (12, 13). This suggests that the majority of Her2-overexpressing tumors have intrinsic resistance to trastuzumab. Combining trastuzumab with paclitaxel (14, 15) or docetaxel (16) increases response rates, but most patients who achieve an initial response to trastuzumab-based regimens will develop resistance within 1 year (15). Efficacy can be further improved with the addition of platinum compounds in the advanced disease setting (17), but resistance likely occurs as well. In the neoadjuvant setting, trastuzumab-containing regimens result in >50% complete pathologic response, implying that there remains a substantial proportion of Her2-overexpressing cancer cells that are resistant to such therapies (18).
Translational Relevance

Despite robust response rates to trastuzumab, either as monotherapy or in combination with conventional chemotherapy, resistance almost inevitably arises within 1 year of initial response. Understanding the mechanisms of such resistance is the first step toward development of eventual interventions that will prevent, delay, or overcome resistance. A common feature of most trastuzumab resistance is constitutive signaling through the phosphoinositide 3-kinase/Akt pathway, but there are multiple mechanisms by which such signaling can be achieved. We have a cell-based model of trastuzumab resistance in which dysregulation of the protein kinase A pathway appears to be a key molecular mechanism by which phosphoinositide 3-kinase/Akt signaling is sustained in the presence of trastuzumab. Studies using this model system and preliminary analysis of clinical specimens suggest that components of the protein kinase A regulatory network could be novel targets for intervention to improve the efficacy of Her2-targeted therapy.

Several mechanisms of intrinsic and acquired resistance have been proposed, including disruption of receptor-antibody interaction (19, 20), compensatory signaling by other Her family receptors (21), signaling by the insulin-like growth factor I receptor (22, 23), and loss of PTEN and mutation of PIK3A, the gene that codes for the p110α catalytic subunit of PI3K (24–26). Although the relative importance of these mechanisms in patient populations has yet to be defined and novel mechanisms may still be discovered, a common feature of these mechanisms is sustained signal transduction predominantly through the PI3K/Akt pathway in the presence of trastuzumab.

An underexplored mechanism of PI3K/Akt regulation is the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway. PKA is an intracellular serine/threonine kinase that plays a diverse role in cell growth and differentiation and it has been shown to intersect with the PI3K/Akt signaling pathway in some cell types (27, 28). Its involvement in breast cancer and resistance to hormone therapy and chemotherapy have been postulated (29–32), but PKA has not been implicated previously in trastuzumab resistance. The regulation of PKA catalytic function is complex, but primary control is mediated by type I or II regulatory subunits that form complexes with the PKAc catalytic subunit. In mammals, there are two type I isoforms (RIα and RIβ) and two type II isoforms (RIIα and RIIβ), each encoded by a unique gene (33). When associated with these regulatory subunits, PKAc activity is repressed, whereas cAMP disrupts the holoenzyme complex and activates PKAc (33). PKAc can also be regulated by modulating the relative levels of type I and II regulatory subunits, even at basal concentrations of intracellular cAMP. In general, type I PKA (bound to type I regulatory subunits) is predominantly associated with high proliferation and malignancy, whereas type II PKA (bound to type II regulatory subunits) is preferentially expressed in nonproliferating, differentiated cells. Changes in the relative amounts of these regulatory subunits can shift the balance toward proliferation or stasis, respectively (34–38).

We have reported previously the selection of clonal variants of Her2-positive BT474 human breast cancer cells (BT/HerR) that are highly resistant to the antiproliferative effects of trastuzumab. Our initial work with these cell lines showed sustained PI3K/Akt signaling and sensitivity to PI3K inhibitors in BT/HerR cells in the presence of trastuzumab, suggesting dysregulation of that pathway as an essential component of trastuzumab-resistant proliferation (39). To understand the mechanism by which the BT/HerR cells are resistant to trastuzumab-mediated Akt dephosphorylation and growth arrest, we analyzed their gene expression profiles by microarray. Here, we report on a group of genes in the PKA signaling network whose expression was altered in BT/HerR cells, relative to parent BT474 cells, with concomitant upregulation of PKA signaling in those cells. We focused on the role of the PKA-RIIα regulatory subunit, which was nearly depleted in BT/HerR clones selected in the presence of 1.0 μmol/L trastuzumab, with no compensatory change in the levels of other PKA subunits. We show that downregulation of PKA regulatory subunit RIα by small interfering RNA (siRNA) conferred partial resistance to trastuzumab-mediated growth arrest and Akt dephosphorylation. Enhanced PKA signaling activity was observed after siRNA-mediated downregulation of PKA-RIIα as well. Moreover, activation of PKA by forskolin caused a stimulation of phospho-Akt levels in parent BT474 in the absence of trastuzumab and protected those cells against Akt dephosphorylation in the presence of trastuzumab. Finally, we analyzed primary tumor samples collected from patients treated with a trastuzumab-containing regimen in the neoadjuvant setting. In the majority of cases analyzed, there appeared to be higher levels of PKA signaling in the surgically resected residual disease remaining after therapy than in the matched pretreatment biopsy specimens collected from the same patients. The potential mechanistic and therapeutic implications of these results are discussed.

Materials and Methods

Cell culture. The human breast cancer cell lines BT474 and SK-Br-3 were obtained from the American Type Culture Collection. BT474 cells were maintained in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin, and RIIβ chem-

RNA analysis. Total cellular RNAs were extracted from BT474 and BT/HerR subclones using the RNasy kit purchased from Qiagen. Details of microarray methods are provided as Supplementary Data. For real-time reverse transcription-PCR, cDNAs were synthesized by random priming using purified total RNA as template. The primer/probe sets and reaction master mix for Taqman real-time PCR were purchased from Applied Biosystems. PCR and real-time quantification were carried out in an auto-lid dual 384-well GeneAmp PCR System (model 9700; Applied Biosystems).

siRNA transfection. siRNA SMARTpools generated by 2'-ACE chemistry targeting PKA-Riα (PKRKA1A gene) and PKA-Riβ (PKRKA2A gene), along with a nontargeting control siRNA, were purchased from Dharmacon Research. siRNA-liposome complexes were prepared using Lipofectamine 2000 in Opti-MEM according to the manufacturer’s
directions. Complexes were added to exponentially growing cells in 60 mm Petri dishes. About 24 h after transfection, medium containing liposome complexes was replaced with normal growth medium. Cells were incubated in fresh medium for an additional 48 h before being assayed for phospho-Akt levels and bromodeoxyuridine (BrdUrd) incorporation.

**Antibodies and Western analysis.** A rabbit monoclonal antibody recognizing Ser473-phospho-Akt was purchased from Cell Signaling Technology. A rabbit polyclonal antibody recognizing total Akt was purchased from Santa Cruz Biotechnology. Monoclonal antibodies recognizing PKA-RII, PKA-RII, or PKAc were purchased from BD Bioscience. Cell samples were dissolved and sonicated in 2× Laemmli sample buffer. After boiling for 5 min, equal amounts of total protein, as determined by the RC-DC Protein Assay kit purchased from Bio-Rad, were loaded onto a 10% SDS-polyacrylamide gel and separated proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and incubated with primary antibody in the blocking buffer. After incubation with a peroxidase-conjugated anti-mouse IgG secondary antibody, the protein of interest was detected using an ECL kit purchased from GE Healthcare. For repeated antibody probing, the membrane was stripped with a Western blot stripping buffer purchased from Pierce. Western hybridization images were digitized by a high-resolution scanner and the densities of individual bands were measured by ImageQuant 5.2 (GE Healthcare).

**Electrophoretic mobility shift assay.** Nuclear extracts were prepared using CelLytic NuCLEAR Extraction kit (Sigma). Electrophoretic mobility shift assay was done using an assay kit purchased from Promega according to the manufacturer's instructions. Briefly, double-stranded oligonucleotide containing a consensus CREB response element (CRE) was labeled with [γ-32P]ATP by end-labeling. DNA-protein binding reactions were done by incubating 5 μg nuclear protein with excess 32P-labeled CRE oligonucleotide in the buffer supplied with the assay kit. For competition binding, 1 pmol of an unlabeled CRE oligonucleotide or an unlabeled nonspecific oligonucleotide was added. After incubation at room temperature for 20 min, binding reactions were resolved on a 4% native polyacrylamide gel. The gel was then dried on to Whatman paper and radioactivity was visualized by autoradiography. The autoradiograph was digitized with a high-resolution scanner and the densities of individual bands were measured by ImageQuant 5.2 (GE Healthcare).

**BrdUrd incorporation assay.** Cells were treated with trastuzumab or PBS for 12 h and then incubated in 10 μmol/L BrdUrd for an additional 18 h in the continuous presence of trastuzumab or PBS. Cells were detached with trypsin and fixed in Cytofix/Cytoperm buffer according to the manufacturer's instructions (BD Bioscience). Fixed cells were treated with DNase to expose incorporated BrdUrd and stained with FITC-conjugated anti-BrdUrd antibody (BD Bioscience) for 1 h at room temperature. Samples were analyzed by flow cytometry to quantify the amount of BrdUrd incorporation. Percentages of FITC-positive cells were determined by analysis with FlowJo software. Statistical analysis was conducted using two-tailed t tests.

**Analysis of clinical samples.** Pretreatment core biopsies and post-treatment surgical specimens were obtained from patients participating in the City of Hope institutional review board–approved protocol 05015. "Randomized Phase II study of docetaxel, Adriamycin, and Cyclophosphamide (TAC) versus Adriamycin/Cyclophosphamide followed by Abraxane/carboplatin (ACAC) +/- trastuzumab as neoadjuvant therapy for patients with stage I-II breast cancer (NCT020925893)." Eligible patients with stage II to III Her2-positive breast cancer were treated with doxorubicin plus cyclophosphamide (every 2 weeks for four cycles) followed by carboplatin plus nab-paclitaxel (Abraxane, weekly for 3 weeks, 1 week off for three cycles) and trastuzumab (loading dose of 4 mg/kg and then weekly at 2 mg/kg for 12 weeks). Definitive surgical intervention was carried out within 4 weeks of the final dose of trastuzumab. Thirty-four Her2-positive patients were enrolled in the trial, with 7 patients having evaluable residual disease at the time of surgery. Per protocol instructions, core biopsy (pretreatment) and surgical specimens (post-treatment from patients with sufficient amounts of residual tumor) were collected and processed for formalin fixation and paraffin embedding in a time frame that would preserve the integrity of phosphoprotein and protein epitopes.

Immunohistochemistry was done on 5-μm-thick serial sections prepared from formalin-fixed, paraffin-embedded tissue using the following rabbit monoclonal antibodies and dilutions: phospho-CREB (Ser133; 87G3; Cell Signaling Technology), 1:50 dilution; CREB (48H2; Cell Signaling Technology), 1:300 dilution; and PKA-RIs (1Y16; Abcam), 1:150 dilution. Tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Samples were then quenched in 3% hydrogen peroxide. For heat-induced epitope retrieval, the sections for phospho-CREB and CREB were steamed with Diva Decloaker buffer, pH 6 (Biocare Medical); for PKA-RIs, the sections were steamed with 1 mol/L EDTA, pH 8 (Lab Vision). Slides were blocked for 10 min using Protein Block from DAKO, and slides were incubated with primary antibody overnight at 4°C. The next day, slides were brought to a DAKO Autostainer universal staining system and developed with the EnVision+ horseradish peroxidase system for detection of rabbit primary antibodies. Counterstaining was with 50% Mayer's hematoxylin (DAKO) for 3 min. Stained slides were scored according to intensity of staining (weak, moderate, or strong) and percentage of tumor cells staining positive for each antigen.

**Results**

Gene expression profiling implicates the PKA signaling network in trastuzumab resistance. We reported previously on the isolation and initial characterization of BT/HerR cell lines selected for their resistance to trastuzumab at 1.0 or 0.2 μmol/L (39). A salient feature of BT/HerR cells is their sustained phospho-Akt levels and Akt activity in the presence of trastuzumab. To address the mechanism by which BT/HerR cells and the PI3K/Akt signaling pathway became resistant to trastuzumab, we analyzed gene expression profiles of two clones (BT/HerR 1.0C and BT/HerR 1.0E) that were selected in 1.0 μmol/L trastuzumab and two clones (BT/HerR 0.2D and BT/HerR 0.2J) that were selected in 0.2 μmol/L trastuzumab in comparison with the trastuzumab-sensitive BT474 parent cells. Details of this analysis are provided as Supplementary Data. Raw microarray data are deposited in the Gene Expression Omnibus database (accession no. GSE15043).

In general, the gene expression profiles of the BT/HerR clones that were initially selected in the same trastuzumab concentration (1.0 or 0.2 μmol/L) were highly similar to each other, with a correlation coefficient of 0.83 for clones BT/HerR 1.0C and BT/HerR 1.0E and a correlation efficient of 0.96 for clones BT/HerR 0.2D and BT/HerR 0.2J (Supplemental Fig. S1). In contrast, expression profiles of the 1.0 and 0.2 μmol/L clones selected under different trastuzumab concentrations shared little similarity. These profiles suggest that clones selected in the same trastuzumab concentration are likely to share similar molecular mechanisms of trastuzumab resistance, whereas clones selected in different trastuzumab concentrations may have acquired resistance by different mechanisms, albeit mechanisms that may overlap or converge on the same central pathway of resistance. The remainder of this report focuses on the molecular mechanism of trastuzumab resistance found in BT/HerR 1.0 clones.

To narrow the search field for the gene(s) responsible for trastuzumab resistance in BT/HerR cells, we performed a pathway analysis to group potential candidate genes based on their relationship with established signaling pathways using
a Web-based Ingenuity database (Ingenuity Systems) and literature searches. This pathway-centric approach led to a group of genes that are directly or indirectly involved in PKA signaling (Table 1). Specifically, the genes encoding PKA-RIIα and PKA-RIIβ subunits were downregulated 2- to 15-fold in BT/HerR<sup>1.0</sup> clones, with no compensatory changes in other PKA subunits, including any of the isoforms of PKAc. In addition, expression of the PKIG gene, whose product acts as an endogenous inhibitor of PKA (40), was downregulated (2- to 4-fold) in all BT/Her<sup>R</sup> clones analyzed. Finally, there were changes in the expression of two genes involved in phosphatase regulation and likely a part of the PKA regulatory pathway: the PPP1R1B gene (41) was highly upregulated in BT/Her<sup>R</sup> 1.0 clones (9- to 50-fold) and the PPP1R3C gene (42) was significantly downregulated (3- to 4-fold) in these clones.

Collectively, these gene expression changes suggested to us that the PKA pathway should be upregulated in BT/Her<sup>R</sup> 1.0 cells. To test this hypothesis, we performed electrophoretic mobility shift assay analysis on nuclear extracts derived from BT474, BT/Her<sup>R</sup> 1.0C, and BT/Her<sup>R</sup> 1.0E cells using a double-stranded oligonucleotide containing a consensus CRE sequence as probe. Nuclear extracts from BT/Her<sup>R</sup> 1.0C and BT/Her<sup>R</sup> 1.0E clones contained 2- to 4-fold higher CRE-binding activity than the parent BT474 cells (see Fig. 4). Although we do not know if this enhanced CRE-binding activity is mediated directly through the PKA pathway, the increased activity of CREB, a nuclear target of the cAMP/PKA signaling pathway (43), is compatible with activation of PKA in BT/Her<sup>R</sup> 1.0 cells.

Given the apparent upregulation of PKA signaling and downregulation of PKA-RIIα and PKA-RIIβ expression in BT/Her<sup>R</sup> 1.0 cells and the known effect of type II PKA regulatory subunits on cell growth and differentiation, the remainder of this report focuses on the PKA regulatory subunits and PKA itself. The role in trastuzumab response of other PKA pathway genes is reported elsewhere (ref. 44; see also Discussion).

**PKA-RIIα downregulation in BT/Her<sup>R</sup> 1.0 cells.** To validate the microarray data, we performed quantitative real-time reverse transcription-PCR analysis of the four PKA regulatory subunits in BT474 and BT/Her<sup>R</sup> clones. Although the microarray results suggested that both PKA-RIIα and PKA-RIIβ genes were downregulated in clones BT/Her<sup>R</sup> 1.0C and BT/Her<sup>R</sup> 1.0E, reverse transcription-PCR analysis indicated that PKA-RIIα was the major type II isoform expressed in BT474 and BT/Her<sup>R</sup> cells. Expression of PKA-RIIβ was extremely low even in parent BT474 cells (data not shown). Similarly, PKA-RIe was the main type I regulatory subunit gene expressed in these clones. As a result, our subsequent studies focused on the RIIα and RIIβ isoforms.

Consistent with the microarray data, real-time reverse transcription-PCR and Western analysis confirmed that PKA-RIIα was nearly depleted in BT/Her<sup>R</sup> 1.0C and BT/Her<sup>R</sup> 1.0E clones relative to levels in parent BT474 cells (Fig. 1). In contrast, levels of PKA-RIIα and PKAc itself were not significantly different in BT/Her<sup>R</sup> clones versus the parent BT474 cells (Fig. 1B), which is also consistent with the microarray data. Because of the connection to trastuzumab resistance, we wanted to determine if expression of the PKA-related proteins changed in response to trastuzumab in either parent BT474 cells or BT/Her<sup>R</sup> clones. We incubated cells with or without the antibody for 1 to 4 h and performed Western analysis on total cell lysates. Trastuzumab had little or no effect on the levels of PKA-RIe, PKA-RIIIα, or PKAc proteins over the 4-h time frame in either parent BT474 or resistant BT/Her<sup>R</sup> cells (Fig. 1B). As observed previously (39), trastuzumab caused an almost complete dephosphorylation of Akt in parent BT474 cells within 2 h but had no significant effect on phospho-Akt levels in BT/Her<sup>R</sup> 1.0C and BT/Her<sup>R</sup> 1.0E clones (Fig. 1B).

**PKA-RIIα downregulation confers partial trastuzumab resistance.** To determine if PKA-RIIα downregulation is sufficient for conferring trastuzumab resistance in BT474 cells, we transfected these cells with a siRNA (siRIIα) targeting the human PKA-RIIα mRNA and looked at effects on cell proliferation and phospho-Akt levels. Cells transfected with a nontargeting siRNA (siControl) were used as control. Because trastuzumab is known to cause G<sub>1</sub>-S cell cycle arrest and inhibition of DNA synthesis in BT474 cells (45), we used a BrdUrd incorporation assay, a measure of DNA synthesis, to determine the effect of PKA-RIIα knockdown on trastuzumab-mediated growth arrest. Trastuzumab caused a significant reduction in the percentage of BrdUrd-positive cells in BT474 cells transfected with siControl but had only about half the effect in cells depleted of PKA-RIIα (Fig. 2A). The difference in response to trastuzumab between siRNA-transfected cells and siControl-transfected cells was statistically significant ($P = 0.0015$). Western analysis

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<th>Symbol</th>
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NOTE: >1 | >-0.4, <0.4 | <1 |
confirmed a significant reduction in the level of PKA-RIIα protein in BT474 cells transfected with siRIIα compared with the level in cells transfected with siControl (Fig. 2B).

As a further test of the role of PKA-RIIα in trastuzumab resistance, we analyzed the effect of its downregulation on intracellular phospho-Akt levels. Trastuzumab caused an almost complete dephosphorylation of Akt in BT474 cells transfected with siControl, consistent with earlier results, but cells transfected with siRIIα were able to maintain a significant level of intracellular phospho-Akt in the presence of trastuzumab (Fig. 3A). These data are consistent with the BrdUrd incorporation data suggesting that PKA-RIIα downregulation conferred partial resistance to trastuzumab-mediated effects.

We also determined if downregulation of PKA-Rα would confer trastuzumab resistance, although microarray and Western analysis indicated that this regulatory subunit was not significantly downregulated in BT/Her² cells. We transfected parent BT474 cells with a siRNA (siRlα) targeting PKA-Rlα and observed a significant reduction in PKA-Rlα protein level 72 h after siRlα transfection (Fig. 3B). In contrast to what was observed when PKA-RIIs was downregulated, downregulation of PKA-Rlα had no effect on trastuzumab-mediated Akt dephosphorylation (Fig. 3B). Instead, PKA-Rlα downregulation caused a small but reproducible increase in the basal level of intracellular phospho-Akt in the absence of any trastuzumab. Taken together, the results shown in Fig. 3 suggest that downregulation of PKA-Rlα conferred at least partial resistance to trastuzumab-mediated effects.

BT474 cells are positive for estrogen receptor and progesterone receptor expression. To determine if downregulation of PKA-Rlα might play a role in conferring trastuzumab resistance in cells that do not express these receptors, we transfected the Her2-positive, estrogen receptor/progesterone receptor–negative SK-Br-3 human breast cancer cell line with siRlα or siControl siRNAs. As with the BT474 cells, trastuzumab caused substantial dephosphorylation of Akt in SK-Br-3 cells transfected with...
siControl, whereas cells transfected with siRIIα were able to maintain a significant level of intracellular phospho-Akt in the presence of trastuzumab (Fig. 3C).

Finally, to confirm that PKA-RIIα downregulation translated into an effect on PKA activity, we performed electrophoretic mobility shift assay analysis on nuclear extracts derived from cells transfected with siRIIα using BT474, BT/HerR1.0C, and BT/HerR1.0E cells as comparison (Fig. 4). As indicated earlier, nuclear extracts from BT/HerR1.0C and BT/HerR1.0E clones contained 2- to 4-fold higher CRE-binding activity than the parent BT474 cells. Likewise, siRIIα-mediated downregulation of PKA-RIIα in BT474 cells enhanced CRE-binding activity by 2-fold.

PKA activation confers resistance to trastuzumab-mediated Akt dephosphorylation. The sum total of PKA-related gene expression changes in BT/HerR1.0 cells (Table 1) and the effects of PKA-RIIα downregulation in BT474 cells suggested to us that dysregulation (stimulation) of PKA activity might be an underlying mechanism of trastuzumab resistance in BT/HerR cells. To test this hypothesis, we treated trastuzumab-sensitive BT474 cells and trastuzumab-resistant BT/HerR cells with 10 μmol/L forskolin, an adenylyl cyclase activator, to increase the level of intracellular cAMP, dissociate both type I and II PKA complexes, and stimulate PKA enzyme activity. We then analyzed the effect of forskolin on basal phospho-Akt levels and trastuzumab-mediated Akt dephosphorylation. Western analysis revealed that forskolin caused an increase in basal intracellular phospho-Akt levels in the absence of trastuzumab in all three cell lines tested (Fig. 5A), consistent with the observation that siRNA-mediated disruption of the type I PKA complex resulted in increased basal phospho-Akt levels (Fig. 2). In the presence of trastuzumab, forskolin again produced a partial resistance to trastuzumab-mediated Akt dephosphorylation in BT474 cells, with no incremental protective effect against dephosphorylation in the resistant BT/HerR1.0 cells (Fig. 5A). Similar results were obtained in the Her2-positive, estrogen receptor/progesterone receptor-negative SK-Br-3 cell line (Fig. 5B).

PKA signaling in primary breast cancer. Our analysis of cell lines suggested that PKA signal transduction is upregulated as an adaptive response to trastuzumab and that this adaptation confers a selective growth advantage (resistance) in the presence of the drug. To determine if this same adaptation might occur in breast cancer patients, we obtained formalin-fixed specimens under institutional review board–approved protocol 05015, a protocol in which patients with stage II to III breast cancer were treated with trastuzumab plus chemotherapy in the neoadjuvant setting. Of 34 Her2-positive patients in the study, 7 had sufficient amounts of residual disease at the time of surgery to permit comparison of PKA signaling proteins in post-treatment surgical samples relative to pretreatment biopsy samples collected from the same patients. To assess the PKA signaling activity in these specimens, we used immunohistochemistry to measure levels of the active, phosphorylated form of CREB (phospho-CREB). Total CREB and PKA-RIIα levels were also evaluated.
We observed three patterns of protein expression, with representative images of each shown in Fig. 6: (a) two of the seven patient sample sets exhibited more intense phospho-CREB and less intense PKA-RIIα staining in the post-treatment specimens relative to their pretreatment counterparts (Fig. 6, top); (b) three sets exhibited more intense phospho-CREB but no difference in PKA-RIIα staining in the post-treatment versus pretreatment samples (Fig. 6, middle); and (c) two sets exhibited no difference or possibly a decrease in phospho-CREB intensity in the post-treatment samples, relative to pretreatment, with essentially no difference in PKA-RIIα between post-treatment and pretreatment samples (Fig. 6, bottom). It is interesting to note that the cases in this last group had very little observable tumor in the surgical samples available for analysis. Long-term outcome data were not yet available for the patients included in this study, so it remains to be seen whether any of these staining patterns will be associated with progression or survival, but these initial results suggest that activation of PKA signaling (phospho-CREB), either through downregulation of PKA-RIIα or through other mechanisms, might be part of the adaptive/resistance response to trastuzumab-containing therapy in breast cancer patients.

Discussion

To understand the mechanism by which breast cancer cells acquire resistance to trastuzumab, we selected several BT/HerR clones that are highly resistant to trastuzumab by culturing the Her2-dependent, trastuzumab-sensitive BT474 human breast cancer cells in the presence of 0.2 or 1.0 μmol/L trastuzumab for 5 to 6 months (39). To determine the molecular mechanisms conferring trastuzumab resistance in BT/HerR clones, we analyzed the gene expression profiles in these cells by microarray. The overall profiles of two BT/HerR clones selected in 1.0 μmol/L trastuzumab were highly correlated with each other as were those of two clones selected in 0.2 μmol/L trastuzumab. In contrast, there was little correlation between the 1.0 and 0.2 μmol/L trastuzumab profiles. This observation suggests that BT/HerR clones selected under different trastuzumab concentrations most likely acquired trastuzumab resistance through different molecular mechanisms. Indeed, downregulation of PRKAR2A and overexpression of PPP1R1B, both of which are at least partly responsible for trastuzumab resistance in the two BT/HerR 1.0 clones selected in 1.0 μmol/L trastuzumab (this report and ref. 44), were not observed in the two BT/HerR 0.2 clones selected in 0.2 μmol/L trastuzumab (see microarray data deposited in Gene Expression Omnibus database, accession no. GSE15043), although both types of clones have the general phenotype of sustained Akt phosphorylation in the presence of trastuzumab (39). The molecular events leading to sustained signaling in BT/HerR 0.2 clones remain to be determined.

In the current study, we show that the PKA signaling pathway is significantly activated in trastuzumab-resistant BT/HerR 1.0 cells and that activation of PKA signaling with forskolin is able to confer resistance to trastuzumab-mediated Akt dephosphorylation. Moreover, downregulation of PKA-RIα, but not PKA-RIβ, appears to be partially responsible for PKA activation and for conferring trastuzumab resistance. This is consistent with published reports that type I and II PKA regulatory subunits exert different effects on cell growth and survival in breast cancer cells. The type II regulatory subunits are preferentially expressed in differentiated nonproliferative tissues, whereas enhanced expression of type I regulatory subunits is found in tumor cells as well as cells exposed to mitogenic stimulation, and it has been correlated with poor prognosis in breast cancer patients (34–38). Downregulation of either regulatory subunit should alter the ratio of type I to II PKA and might lead to an increase in free PKAα level and PKA activity even in the absence of any stimulatory trigger from cAMP. Further work is needed, however, to understand the mechanism by which downregulation of one subunit (PKA-RIα) but not the other (PKA-RIβ) exerts an effect on cellular sensitivity to trastuzumab. Distinct subcellular localization (46) and differential affinity for cAMP between type I and II regulatory subunits (47, 48) have been proposed to account for their differential effects on cell proliferation, so these should be explored as possible reasons for differential effects on resistance as well. More recently, activation of type II PKA by cAMP-independent mechanisms (49–51) has been reported and might also account for the differences observed in our studies. Regardless of the mechanistic details, several approaches have been explored to modulate the intracellular balance between type I and II PKAs for treating breast cancers (34, 52), and these might also affect response to trastuzumab and possibly other therapies.

Fig. 4. Effect of PKA-RIα downregulation on CRE-binding activity. Nuclear extracts were prepared from parent BT474 cells (BT), BT/HerR 1.0C, and BT/HerR 1.0E clones and from BT cells transfected with a nontargeting control siRNA (siControl) or a siRNA targeting PKA-RIα (siRIα). Nuclear extracts (5 μg) were subjected to electrophoretic mobility shift assay using a 32P-labeled oligonucleotide probe containing a CRE (Promega) in the absence of unlabeled competitor oligonucleotide or in the presence of 1 μmol of an unlabeled CRE or nonspecific (control) competitor oligonucleotide. Products of the binding reactions were separated on a 4% native polyacrylamide gel and visualized by autoradiography. CRE-binding activities were quantified by measuring the densities of individual bands on the autoradiograph. The number under each lane indicates the CRE-binding activity in each cell lysate normalized to that in the untreated BT474 cells.
Our preliminary analysis of clinical specimens suggests that enhanced PKA signaling may be one type of adaptive response to trastuzumab-containing therapy in patients, at least some of which could be due to downregulation of PKA-RILα (Fig. 6). We cannot definitively state that enhanced PKA signaling in post-treatment samples is a response to trastuzumab per se, given that patients in the study were treated with multiple chemotherapeutic agents, and larger studies will be required to determine the relationship between PKA signaling and clinical outcomes, but the data presented here nevertheless suggest that PKA signaling could be a viable secondary target in a subset of patients on trastuzumab-containing therapy.

There are at least three mechanisms by which enhanced PKA activity might affect trastuzumab resistance (see Fig. 7). First, it is known that the PKA signaling pathway can interact directly with the EGFR signaling pathway (53). Thus, enhanced PKA activity resulting from PKA-RILα downregulation in BT/HerR cells might promote cell growth and proliferation directly by enhancing EGFR signaling. We found previously that BT/HerR cells are more sensitive to the EGFR inhibitor AG1478 in the presence of trastuzumab than in its absence (39), suggesting that BT/HerR clones are more dependent on EGFR when Her2 is shut down. If PKA-mediated activation of EGFR is found to be important for trastuzumab resistance, then simultaneous inhibition of EGFR and Her2, either by combination therapy with trastuzumab and an EGFR inhibitor or by EGFR/Her2 dual kinase inhibitors, might provide a way of treating Her2-positive breast cancers that are resistant to trastuzumab or perhaps minimizing the emergence of such resistance in the first place. Clinical studies to explore these possibilities have been initiated, with promising but incomplete results thus far (54).

A second mechanism by which PKA activation might affect trastuzumab resistance is directly through the PI3K/Akt pathway. It has recently been reported that PKA can phosphorylate the p85 subunit of PI3K, thereby activating PI3K signaling (27). Our previous observations suggested that BT/HerR cells continue to signal through PI3K/Akt in the presence of trastuzumab and are sensitive to inhibition by LY294002 (39). The current work raises the possibility that this sustained PI3K/Akt signaling might be due, at least in part, to dysregulated PKA. Finally, an indirect effect of PKA on phospho-Akt levels could be mediated through protein phosphatase-1 (PP-1), a multifunctional phosphatase that is intimately involved in various signal transduction pathways. In at least some cell types, PKA can downmodulate PP-1 activity and thus potentiate Akt phosphorylation (see below). There are no doubt other means by which PKA can influence PI3K/Akt signaling and/or trastuzumab resistance, so it will be important to determine experimentally the exact mechanism of the effects of PKA.

It is notable that the BT/HerR 1.0 clones were more resistant to trastuzumab-mediated Akt dephosphorylation than parent BT474 cells in which PKA-RILα levels were downregulated, suggesting that other factors also contribute to trastuzumab resistance in BT/HerR cells. Indeed, the PKA pathway interacts with and is regulated by multiple proteins, some of which were coordinately altered along with PKA-RILα in BT/HerR cells (Table 1). Most significantly, PKIG expression was downregulated 2- to 4-fold, PPP1R1B expression was upregulated by as much as 50-fold, and PPP1R3C expression was downregulated 3- to 4-fold in the BT/HerR 1.0 clones. The PKIG gene product, PKIγ, is a negative regulator of PKAs (40), so its downregulation could contribute to enhanced PKA activity in BT/HerR 1.0 clones. PPP1R1B encodes two known transcriptional variants (55), dopamine and AMP-regulated phosphoprotein of 32 kDa (Darpp-32) and truncated Darpp-32 (t-Darpp), both of which are detectable by the same probe sets on microarray chips. Further analysis of BT/HerR 1.0 cells showed that it is the t-Darpp variant whose expression is upregulated, whereas Darpp-32 expression is not significantly changed in these cells, relative to parent BT474 cells (44). Darpp-32 plays an important role in dopamine signaling and is a negative regulator of both PKA and PP-1 (41). The exact function and mechanism of t-Darpp are not known, but its role in trastuzumab resistance has been reported by us and others (44, 56, 57). The PPP1R3C gene product, called protein targeting to glycogen (PTG), is a scaffold protein that promotes PP-1 activity and also modulates at least some aspect of Darpp-32 function (42), thus potentially linking PTG to PKA via the PKA/Darpp-32/PP-1 signaling cascade (41). Again, the role of these other proteins and the mechanism(s) by which they might influence trastuzumab resistance and PI3K/Akt signaling will need to be studied.

The analysis of clinical samples reported here suggests that there are multiple mechanisms by which PKA signaling might be activated in patients as well, because PKA-RILα downregulation was observed in only a subset of samples in which phospho-CREB was elevated. A larger number of pretreatment and
post-treatment tumor samples is required to pursue this line of investigation. Likewise, there are probably other factors unrelated to the PKA network that also contribute to trastuzumab resistance and sustained PI3K/Akt signaling in BT/HerK 1.0 cells and patients, and these will need to be explored in further laboratory and clinical studies as well.

In summary, we present data indicating that aberrant regulation of the PKA pathway can contribute to trastuzumab resistance in Her2-positive breast cancer cells. Activation of PKA itself appears to confer at least partial resistance to trastuzumab. Moreover, PKA signaling appears to be upregulated as an adaptive response in at least some patients treated with a trastuzumab-containing regimen in the neoadjuvant setting. We propose a working model (Fig. 7) by which PKA might influence breast cancer response to trastuzumab therapy. Whatever the mechanism of the effects of PKA, the current study implicates PKA and its regulatory network as potential targets for preventing the emergence of resistance or to improve the response to treatment. 

Fig. 6. Immunohistochemistry of clinical samples. Serial sections made from pretreatment biopsies (Pre) of three Her2-positive breast cancer patients and the residual tumors (Post) surgically removed from the same patients after treatment with a trastuzumab-containing regimen. The sectioned samples were stained with antibodies against phospho-CREB (left), total CREB (middle), or PKA-RIIα (right) as described in Materials and Methods. Top, a case in which phospho-CREB staining was stronger and PKA-RIIα staining was weaker after treatment compared with the corresponding pretreatment sample; middle, a case in which phospho-CREB was stronger but PKA-RIIα was about the same after treatment; bottom, a case in which phospho-CREB was the same or weaker after treatment (see text). Slides were visualized on an Olympus AX70 microscope under a ×10 objective lens. Images were digitized using a Retiga EX color camera from Qimaging and Image Pro Plus imaging software version 6.3 (Media Cybernetics).
Fig. 7. A working model of the role of PKA in trastuzumab resistance. The canonical mechanism of PKA activation is through cAMP-mediated release of regulatory subunits from the catalytic PKAc subunit of the enzyme. Left, release of the RIIα subunit by cAMP (red circles); green arrow, downregulation of PKA-RIIα in trastuzumab-resistant cell lines, also postulated to activate PKAc; right, stimulation of the PI3K/Akt pathway by Her2/Her3 heterodimers, the predominant receptor tyrosine kinase pathway in BT474 cells, and blockage (red X) of the Her2 component of that pathway by trastuzumab; dashed lines and question marks, hypothetical pathways that might be affected by activated PKA in BT/HerR cells to mediate sustained PI3K/Akt signaling and trastuzumab resistance. These include direct activation of EGFR or PI3K and indirect activation of phospho-Akt by inhibition of PP-1 (see text). The activation of PI3K/Akt via EGFR (either as a homodimer or as a heterodimer with Her3) on trastuzumab-mediated inhibition of Her2 is also hypothetical but consistent with our earlier studies of BT/HerR cells (39).

efficacy of trastuzumab-containing regimens delivered in the neoadjuvant or adjuvant setting.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


