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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 one year. BT/Her^R cells are trastuzumab-resistant variants of Her2-positive BT474 breast cancer cells. The salient feature of BT/Her^R cells is failure to down-regulate PI3K/Akt signaling upon trastuzumab binding. The current work addresses the mechanism of sustained signaling in BT/Her^R cells, focusing on the protein kinase A (PKA) pathway.

Experimental Design—We performed microarray analysis on BT/Her^R and BT474 cell lines to identify genes that were up- or down-regulated in trastuzumab resistant cells. Specific genes in the PKA pathway were quantified using RT-PCR and Western hybridization. SiRNA transfection was used to determine the effects of gene knockdown on cellular response to trastuzumab. Electrophoretic mobility shift assays were used to measure cAMP-responsive element binding activity under defined conditions. Immunohistochemistry was used to analyze protein expression in clinical samples.

Results—BT/Her^R cells had elevated PKA signaling activity and several genes in the PKA regulatory network had altered expression in these cells. Down-regulation of one such gene, the PKA-RIIα 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.

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TRANSLATIONAL RELEVANCE

Despite robust response rates to trastuzumab, either as monotherapy or in combination with conventional chemotherapy, resistance almost inevitably arises within one year of initial response. Understanding the mechanisms of such resistance is the first step towards development of eventual interventions that will prevent, delay or overcome resistance. A common feature of most trastuzumab resistance is constitutive signaling through the PI3K/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 (PKA) pathway appears to be a key molecular mechanism by which PI3K/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 PKA regulatory network could be novel targets for intervention to improve the efficacy of Her2-targeted therapy.

Keywords

PKA; PKA-RIIα; Herceptin; breast cancer; drug resistance

INTRODUCTION

The Her2 (*erb*B2/*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 20-25% 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 Inc.), 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 down-modulation of PI3K/Akt and/or Ras/MAPK 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 12-34% 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 a year (15). Efficacy can be further improved with the addition of platinum compounds in the advanced disease setting (17), but resistance likely occurs here as well. In the neoadjuvant setting, trastuzumab-containing regimens result in over 50% complete pathological response, implying that there remains a substantial proportion of Her2-overexpressing cancer cells that are resistant to such therapies (18).

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), 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 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 previously been implicated in trastuzumab resistance. The regulation of PKA catalytic function is complex, but primary control is mediated by type I or type 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 type 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 non-proliferating, differentiated cells. Changes in the relative amounts of these regulatory subunits can shift the balance towards proliferation or stasis, respectively (34-38).

We have previously reported the selection of clonal variants of Her2-positive BT474 human breast cancer cells (BT/Her^R) that are highly resistant to the anti-proliferative effects of trastuzumab. Our initial work with these cell lines demonstrated sustained PI3K/Akt signaling and sensitivity to PI3K inhibitors in BT/Her^R 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/Her^R 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/Her^R 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/Her^R clones selected in the presence of 1.0 μM trastuzumab, with no compensatory change in the levels of other PKA subunits. We demonstrate that down-regulation of PKA regulatory subunit RIIα by siRNA conferred partial resistance to trastuzumab-mediated growth arrest and Akt dephosphorylation. Enhanced PKA signaling activity was observed after siRNA-mediated down-reguation 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 pre-treatment 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 (Rockville, MD). BT474 cells were maintained in DMEM with 10% FBS and 1% penicillin/streptomycin in 5% CO_2. SK-Br-3 cells were maintained in McCoy's Medium 5A with 10% FBS, 1% penicillin/streptomycin, and L-glutamine in 5% CO_2. BT/Her^R clones, previously derived from BT474 cells after a six-month selection in the continuous presence of trastuzumab (39), were maintained in the same culture conditions as the BT474 cells. They were tested regularly in trastuzumab-containing medium to ensure their drug resistance.

RNA analysis

Total cellular RNAs were extracted from BT474 and BT/Her^R subclones using the RNeasy kit purchased from Qiagen (Valencia, CA). Details of microarray methods are provided as Supplementary Data. For real time RT-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 (Foster City, CA). 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 (PRKAR1A gene) and PKA-RIIα (PRKAR2A gene), along with a non-targeting control siRNA, were purchased

from Dharmacon Research (Lafayette, CO). 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 hours after transfection, medium containing liposome complexes was replaced with normal growth medium. Cells were incubated in fresh medium for an additional 48 hours before being assayed for phospho-Akt levels and bromodeoxyuridine (BrdU) incorporation.

Antibodies and Western analysis

A rabbit monoclonal antibody recognizing Ser^{473}-phosphorylated Akt was purchased from Cell Signal Technologies (Danvers, MA). A rabbit polyclonal antibody recognizing total Akt was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies recognizing PKA-RIα, PKA-RIIα, or PKAc were purchased from BD Bioscience (San Jose, CA). Cell samples were dissolved and sonicated in 2x Laemmli sample buffer. After boiling for five minutes, equal amounts of total protein, as determined by the *RC DC* Protein Assay kit purchased from Bio-Rad (Hercules, CA), were loaded onto a 10% SDS-polyacrylamide gel and separated proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat 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 (Piscataway, NJ). For repeated antibody probing, the membrane was stripped with a Western blot stripping buffer purchased from Pierce (Rockford, IL). Western hybridization images were digitized by a high-resolution scanner and the densities of individual bands were measured by ImageQuant^{MT} 5.2 (GE Healthcare, Piscataway, NJ).

Electrophoretic mobility shift assay

Nuclear extracts were prepared using CelLytic™ NuCLEAR™ Extraction Kit (Sigma, Saint Louis, MO). Electrophoretic mobility shift assay (EMSA) was done using an assay kit purchased from Promega (Madison, WI), according to manufacturer's instructions. Briefly, double-stranded oligonucleotide containing a consensus CREB response element (CRE) was labeled with γ-^{32}P ATP by end-labeling. DNA-protein binding reactions were performed by incubating 5 μg of nuclear protein with excess ^{32}P-labeled CRE oligonucleotide in the buffer supplied with the assay kit. For competition binding, 1 pmol of an unlabeled CRE oligonucleotide or an unlabeled non-specific 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 onto 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^{MT} 5.2 (GE Healthcare, Piscataway, NJ).

BrdU incorporation assay

Cells were treated with trastuzumab or phosphate buffered saline (PBS) for twelve hours and then incubated in 10 μM BrdU for an additional eighteen hours 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, San Jose, CA). Fixed cells were treated with DNase to expose incorporated BrdU and were stained with FITC-conjugated anti-BrdU antibody (BD Bioscience) for one hour at room temperature. Samples were analyzed by flow cytometry to quantify the amount of BrdU incorporation. Percentages of FITC-positive cells were determined by analysis with FlowJo software (Ashland, OR). Statistical analysis was conducted using two-tailed *t*-tests.

Analysis of clinical samples

Pre-treatment core biopsies and post-treatment surgical specimens were obtained from patients participating in City of Hope's IRB-approved protocol #05015, "Randomized phase II study of docetaxel, adriamycin, and cytoxan (TAC) versus adriamycin/cytoxan, followed by abraxane/carboplatin (ACAC) +/- trastuzumab as neoadjuvant therapy for patients with stage I-III breast cancer (NCT00295893). Eligible patients with stage II-III Her2-positive breast cancer were treated with doxorubicin plus cyclophosphamide (every 2 weeks for 4 cycles) followed by carboplatin plus *nab*-paclitaxel (Abraxane®, weekly for 3 weeks, one week off for 3 cycles) and trastuzumab (loading dose of 4 mg/kg, then weekly at 2 mg/kg for 12 weeks). Definitive surgical intervention was carried out within four weeks of the final dose of trastuzumab. Thirty-four Her2-positive patients were enrolled in the trial, with seven patients having evaluable residual disease at the time of surgery. Per protocol instructions, core biopsy (pre-treatment) 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 timeframe that would preserve the integrity of phosphoprotein and protein epitopes.

Immunohistochemistry was performed 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) (#9198 from Cell Signaling Technology, Danvers, MA), 1:50 dilution; CREB (48H2) (#9197 from Cell Signaling Technology), 1:300; and PKA-R2α (Y116) (#ab32514 from Abcam, Cambridge, MA), 1:150. 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 pCREB and CREB were steamed with Diva Decloaker buffer, pH.6 (Biocare Medical, Concord, CA); for PKA-R2α, the sections were steamed with 1 mM EDTA, pH.8 (Lab Vision Corporation, Fremont, CA). Slides were blocked for 10 minutes using Protein Block from Dako (Carpenteria, CA) and then 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 + HRP 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 previously reported on the isolation and initial characterization of BT/Her^R cell lines selected for their resistance to trastuzumab at 1.0 μM or 0.2 μM (39). A salient feature of BT/Her^R cells is their sustained phospho-Akt levels and Akt activity in the presence of trastuzumab. To address the mechanism by which BT/Her^R cells and the PI3K/Akt signaling pathway became resistant to trastuzumab, we analyzed gene expression profiles of two clones (BT/Her^{R}1.0C and BT/Her^R 1.0E) that were selected in 1.0 μM trastuzumab and two clones (BT/Her^{R}0.2D and BT/Her^R 0.2J) that were selected in 0.2 μM trastuzumab, in comparison to 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 #GSE15043).

In general, the gene expression profiles of the BT/Her^R clones that were initially selected in the same trastuzumab concentration (1.0 μM or 0.2 μM) were highly similar to each other, with a correlation coefficient of 0.83 for clones BT/Her^R 1.0C and BT/Her^R 1.0E and a correlation efficient of 0.96 for clones BT/Her^{R}0.2D and BT/Her^R 0.2J (Supplementary Data Fig. S1). In contrast, expression profiles of the 1.0 μM and 0.2 μM 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/Her^{R}1.0 clones.

To narrow the search field for the gene(s) responsible for trastuzumab resistance in BT/Her^R 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, Redwood City, CA) 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 RIIβ subunits were down-regulated 2- to 15-fold in BT/Her^{R}1.0 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 down-regulated (2- to 4-fold) in all BT/Her^R 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 up-regulated in BT/Her^{R}1.0 clones (9- to 50-fold) and the PPP1R3C gene (42) was significantly down-regulated (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^{R}1.0 cells. To test this hypothesis, we performed EMSA analysis on nuclear extracts derived from BT474, BT/Her^{R}1.0C, and BT/Her^{R}1.0E cells, using a double-stranded oligonucleotide containing a consensus CRE sequence as probe. Nuclear extracts from BT/Her^{R}1.0C and BT/Her^{R}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^{R}1.0 cells.

Given the apparent up-regulation of PKA signaling and down-regulation of PKA-RIIα and PKA-RIIβ expression in BT/Her^{R}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 (44) (also see the Discussion).

PKA-RIIα is down-regulated in BT/Her^{R}1.0 cells

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

Consistent with the microarray data, real-time RT-PCR and Western analysis confirmed that PKA-RIIα was nearly depleted in BT/Her^{R}1.0C and BT/Her^{R}1.0E clones, relative to levels in parent BT474 cells (Fig. 1). In contrast, levels of PKA-RIα and PKAc itself were not significantly different in BT/Her^R clones vs. 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, either in parent BT474 cells or BT/Her^R clones. We incubated cells with or without the antibody for 1-4 hours and performed Western analysis on total cell lysates. Trastuzumab had little or no effect on the levels of PKA-RIα, PKA-RIIα, or PKAc proteins over the four-hour time frame, in either parent BT474 or resistant BT/Her^R cells (Fig. 1b). As previously observed (39), trastuzumab caused an almost complete dephosphorylation of Akt in parent BT474 cells within 2 hours, but had no significant effect on phospho-Akt levels in BT/Her^{R}1.0C and BT/Her^{R}1.0E clones (Fig. 1b).

PKA-RIIα down-regulation confers partial trastuzumab resistance

To determine if PKA-RIIα down-regulation 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 non-targeting siRNA (siControl) were used as control. Since trastuzumab is known to cause G1/S cell cycle arrest and inhibition of DNA synthesis in BT474 cells (45), we used a BrdU 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 BrdU-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 siRIIα-transfected cells and siControl-transfected cells was statistically significant ($p = 0.0015$). Western analysis 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 down-regulation 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 BrdU incorporation data suggesting that PKA-RIIα down-regulation conferred partial resistance to trastuzumab-mediated effects.

We also determined if down-regulation of PKA-RIα would confer trastuzumab resistance, even though microarray and Western analysis had indicated that this regulatory subunit was not significantly down-regulated in BT/Her^R cells. We transfected parent BT474 cells with a siRNA (siRIα) targeting PKA-RIα and observed a significant reduction in PKA-RIα protein level 72 hours after siRIα transfection (Fig. 3b). In contrast to what was observed when PKA-RIIα was down-regulated, down-regulation of PKA-RIα had no effect on trastuzumab-mediated Akt dephosphorylation (Fig. 3b). Instead, PKA-RIα down-regulation 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 down-regulation of PKA-RIIα conferred at least partial resistance to trastuzumab-mediated Akt dephosphorylation in BT474 cells, and that down-regulation of PKA-RIα had a modest stimulatory effect on basal phospho-Akt levels without preventing its inhibition by trastuzumab.

BT474 cells are positive for estrogen receptor (ER) and progesterone receptor (PR) expression. To determine if down-regulation of PKA-RIIα might play a role in conferring trastuzumab resistance in cells that do not express these receptors, we transfected the Her2-positive, ER/PR-negative SK-Br-3 human breast cancer cell line with siRIIα 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 down-regulation translated into an effect on PKA activity, we performed EMSA analysis on nuclear extracts derived from cells transfected with siRIIα, using BT474, BT/Her^{R}1.0C, and BT/Her^{R}1.0E cells as comparison (Fig. 4). As indicated earlier, nuclear extracts from BT/Her^{R}1.0C and BT/Her^{R}1.0E clones contained 2- to 4-fold higher CRE binding activity than the parent BT474 cells. Likewise, siRIIα-mediated down-regulation 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/Her^{R}1.0 cells (Table 1) and the effects of PKA-RIIα down-regulation in BT474 cells suggested to us that dysregulation (stimulation) of PKA activity might be an underlying mechanism of trastuzumab resistance in BT/Her^R cells. To test this hypothesis, we treated trastuzumab-sensitive BT474 cells and trastuzumab-resistant BT/Her^R cells with 10 μM forskolin, an adenylyl-cyclase activator, to increase the level of intracellular cAMP, dissociate both type-I and type-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/Her^{R}1.0 cells (Fig. 5a). Similar results were obtained in the Her2-positive, ER/PR-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 up-regulated 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 IRB 05015, an approved protocol in which patients with stage II-III breast cancer were treated with trastuzumab plus chemotherapy in the neoadjuvant setting. Of 34 Her2-positive patients in the study, seven 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 pre-treatment 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 (pCREB). 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: 1) Two of the seven patient sample sets exhibited more intense pCREB and less intense PKA-RIIα staining in the post-treatment specimens, relative to their pre-treatment counterparts (Fig. 6, top); 2) three sets exhibited more intense pCREB but no difference in PKA-RIIα staining in the post-treatment vs. pre-treatment samples (Fig. 6, middle); and 3) two sets exhibited no difference or possibly a decrease in pCREB intensity in the post-treatment samples, relative to pre-treatment, with essentially no difference in PKA-RIIα between post- and pre-treatment 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 (pCREB), either through down-regulation 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/Her^R 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 μM or 1.0 μM trastuzumab for 5-6 months (39). To determine the molecular mechanisms conferring trastuzumab resistance in BT/Her^R clones, we analyzed the gene expression profiles in these cells by microarray. The overall profiles of two BT/Her^R clones selected in 1.0 μM trastuzumab were highly correlated with each other as were those of two clones selected in 0.2 μM trastuzumab. In contrast, there was little correlation between the 1.0 μM and 0.2 μM trastuzumab profiles. This observation suggests that BT/Her^R clones selected under different trastuzumab concentrations most likely acquired trastuzumab resistance through different molecular mechanisms. Indeed, down-regulation of PRKAR2A and overexpression of PPP1R1B, both of which are at least partly responsible for trastuzumab resistance in the two BT/Her^{R}1.0 clones selected in 1.0 μM trastuzumab (this report and (44)), were not observed in the two BT/Her^{R}0.2 clones selected in 0.2 μM trastuzumab (see microarray data deposited in GEO database, Accession #GSE15043), even though 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/Her^{R}0.2 clones remain to be determined.

In the current study, we demonstrate that the PKA signaling pathway is significantly activated in trastuzumab-resistant BT/Her^{R}1.0 cells and that activation of PKA signaling with forskolin is able to confer resistance to trastuzumab-mediated Akt dephosphorylation. Moreover, down-regulation of PKA-RIIα, but not PKA-RIα appears to be partially responsible for PKA activation and for conferring trastuzumab resistance. This is consistent with published reports that type-I and type-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 non-proliferating 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). Down-regulation of either regulatory subunit should alter the ratio of type-I to type-II PKA and might lead to an increase in free PKAc 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 down-regulation of one subunit (PKA-RIIα) but not the other (PKA-RIα) exerts an effect on cellular sensitivity to trastuzumab. Distinct subcellular localization (46) and differential affinity for cAMP between type-I and type-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 type-II PKAs for treating breast cancers (34,52), and these might also affect response to trastuzumab and possibly other therapies.

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 down-regulation of PKA-RIIα (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 impact 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-RIIα down-regulation in BT/Her^R cells might promote cell growth and proliferation directly by enhancing EGFR signaling. We previously found that BT/Her^R cells are more sensitive to the EGFR inhibitor AG1478 in the presence of trastuzumab than in its absence (39), suggesting that BT/Her^R 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 so far (54).

A second mechanism by which PKA activation might impact 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/Her^R 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 multi-functional phosphatase that is intimately involved in various signal transduction pathways. In at least some cell types, PKA can down-modulate 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 PKA's effects.

It is notable that the BT/Her^{R}1.0 clones were more resistant to trastuzumab-mediated Akt dephosphorylation than parent BT474 cells in which PKA-RIIα levels were down-regulated, suggesting that other factors also contribute to trastuzumab resistance in BT/Her^R cells. Indeed, the PKA pathway interacts with and is regulated by multiple proteins, some of which were coordinately altered along with PKA-RIIα in BT/Her^R cells (Table 1). Most significantly, PKIG expression was down-regulated 2- to 4-fold, PPP1R1B expression was up-regulated by as much as 50-fold, and PPP1R3C expression was down-regulated 3- to 4-fold in the BT/Her^{R}1.0 clones. The PKIG gene product, PKIγ, is a negative regulator of PKAc (40), so its down-regulation could contribute to enhanced PKA activity in BT/Her^{R}1.0 clones. PPP1R1B encodes two known transcriptional variants (55), Darpp-32 (dopamine and adenosine 3′,5′-monophosphate-regulated phosphoprotein of 32 kDa) and t-Darpp (truncated Darpp-32), both of which are detectable by the same probe sets on microarray chips. Further analysis of BT/Her^{R}1.0 cells showed that it is the t-Darpp variant whose expression is up-regulated, 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 the truncated 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, since PKA-RIIα down-regulation was observed in only a subset of samples in which pCREB was elevated. A larger number of

pre- 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/Her^{R}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 up-regulated 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 PKA's effects, the current study implicates PKA and its regulatory network as potential targets for preventing the emergence of resistance or to improve the efficacy of trastuzumab-containing regimens delivered in the neoadjuvant or adjuvant setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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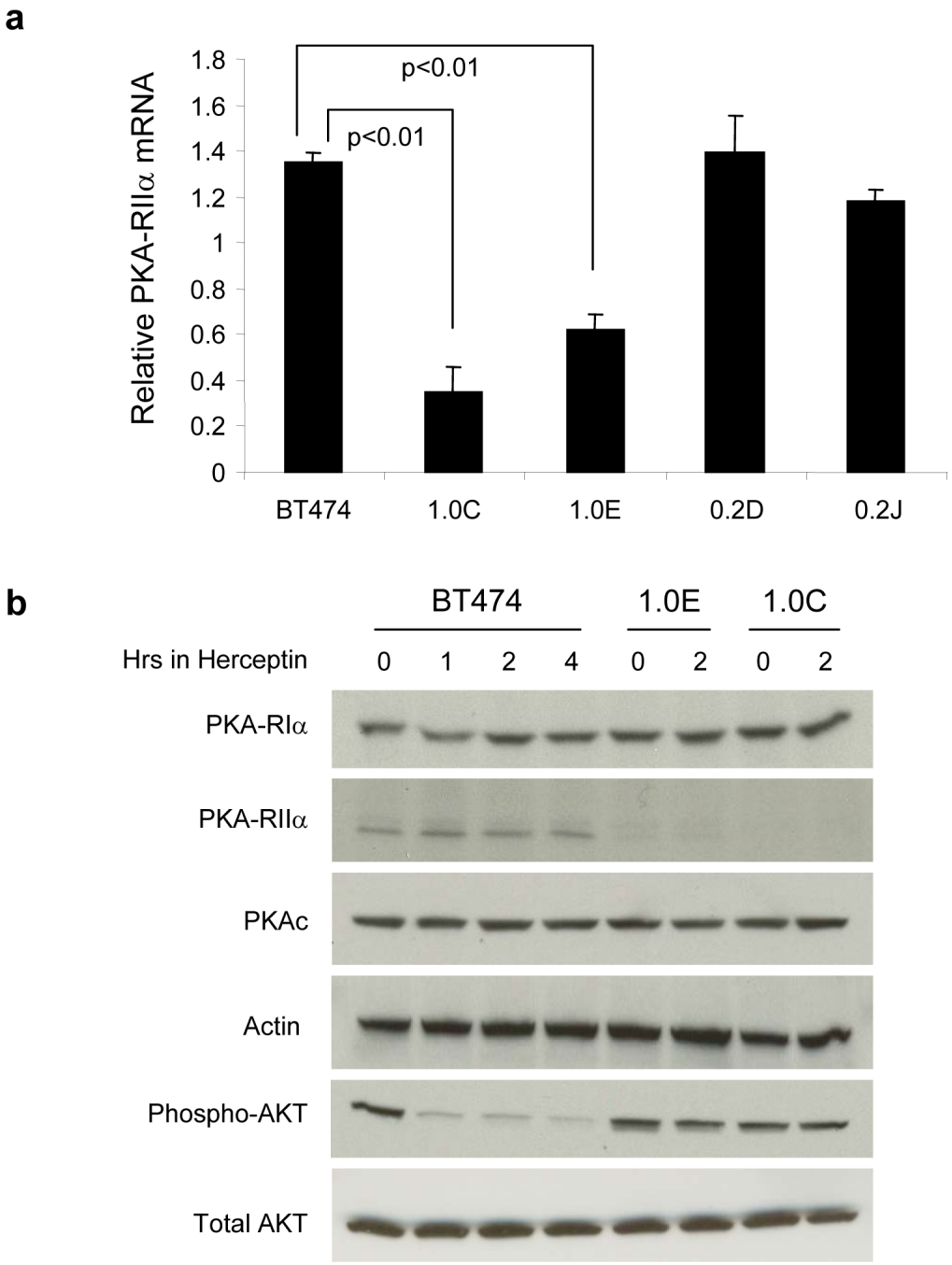

Figure 1.

PKA subunit expression in BT/Her^R clones. (a) PKA-RIIα mRNA expression in BT474 cells and BT/Her^R clones was analyzed by a Taqman assay. Shown are the PKA-RIIα mRNA expression levels in each indicated cell line relative to that in a pool of RNAs used as standard (average of triplicate measurements, ±S.D.). Statistical significance, relative to BT474 cells, was determined by two-tailed *t*-test. (b) Total cellular proteins were extracted from BT474, BT/Her^{R}1.0E (1E), and BT/Her^{R}1.0C (1C) clones before or at the indicated times after the addition of trastuzumab to the growth medium. Expression of PKA-RIα, PKA-RIIα, PKAc, phospho-Akt and total Akt was analyzed by Western hybridization as described in Materials and Methods.

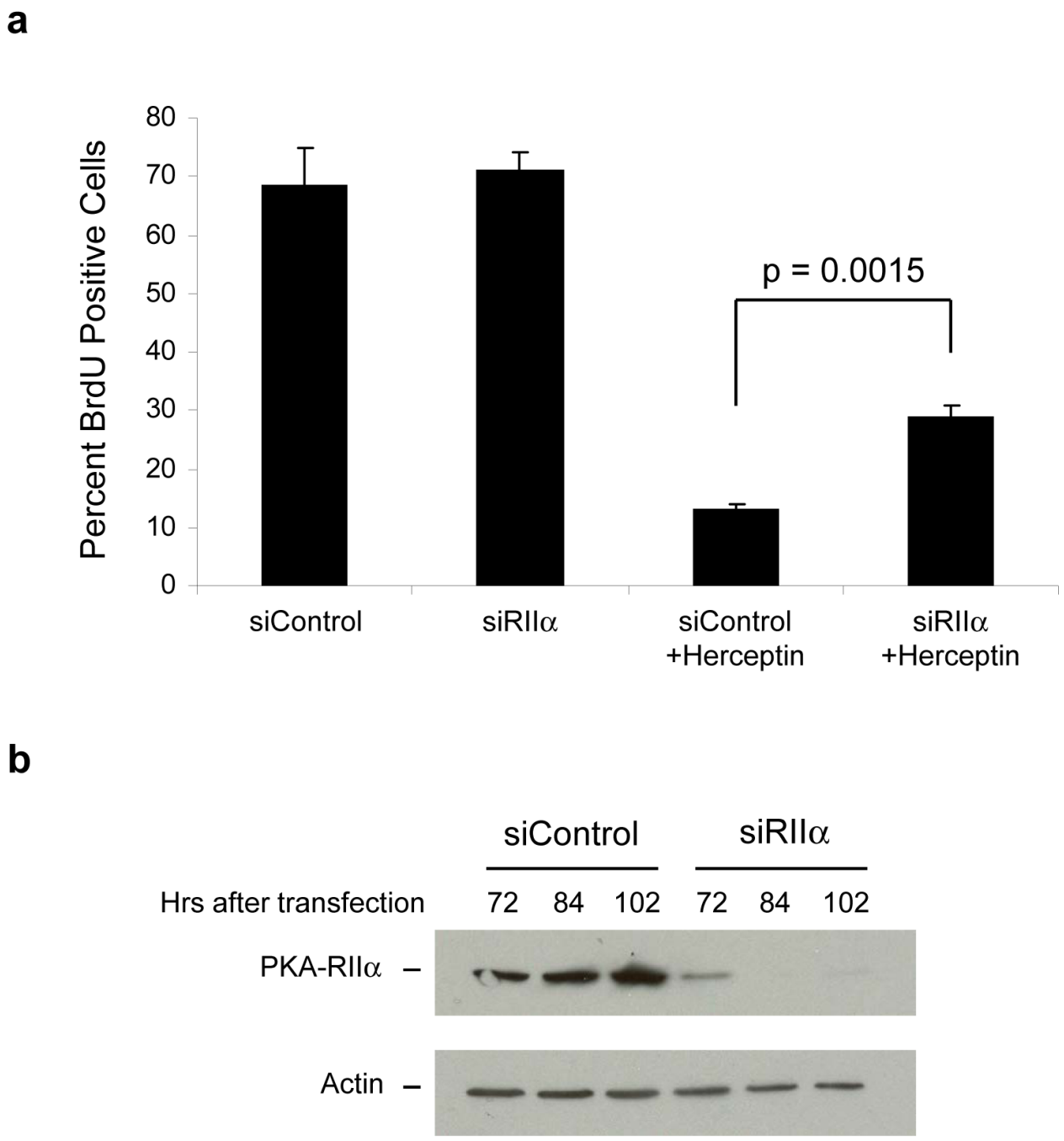

Figure 2.

The effect of PKA-RIIα down-regulation on trastuzumab-mediated growth arrest. Parent BT474 cells were transfected with a siRNA targeting PKA-RIIα (siRIIα) or a control, non-targeting siRNA (siControl). Seventy-two hours post-transfection, cells were incubated for 12 hours in the presence of 1.0 μM trastuzumab or PBS and then incubated in 10 μM bromodeoxyuridine (BrdU) for an additional 18 hours in the continual presence of trastuzumab or PBS. a) BrdU incorporation was measured by flow cytometry after staining with FITC-conjugated anti-BrdU antibody. Percentages of FITC-positive cells were determined by analysis with FlowJo. The percentages of FITC-positive cells from three independently transfected cell samples were averaged and graphed (±S.D.). Statistical significance was

determined by two-tailed *t*-test. b) To ensure downregulation of PKA-RIIα by siRNA during the experimental window, we collected cells lysates from parallel transfected cells at 72, 84, and 102 hours after siRNA transfection and analyzed their PKA-RIIα and actin levels by Western hybridization. The selected time points corresponded to the start of trastuzumab exposure, addition of BrdU, and harvest of cells for flow cytometry analysis, respectively.

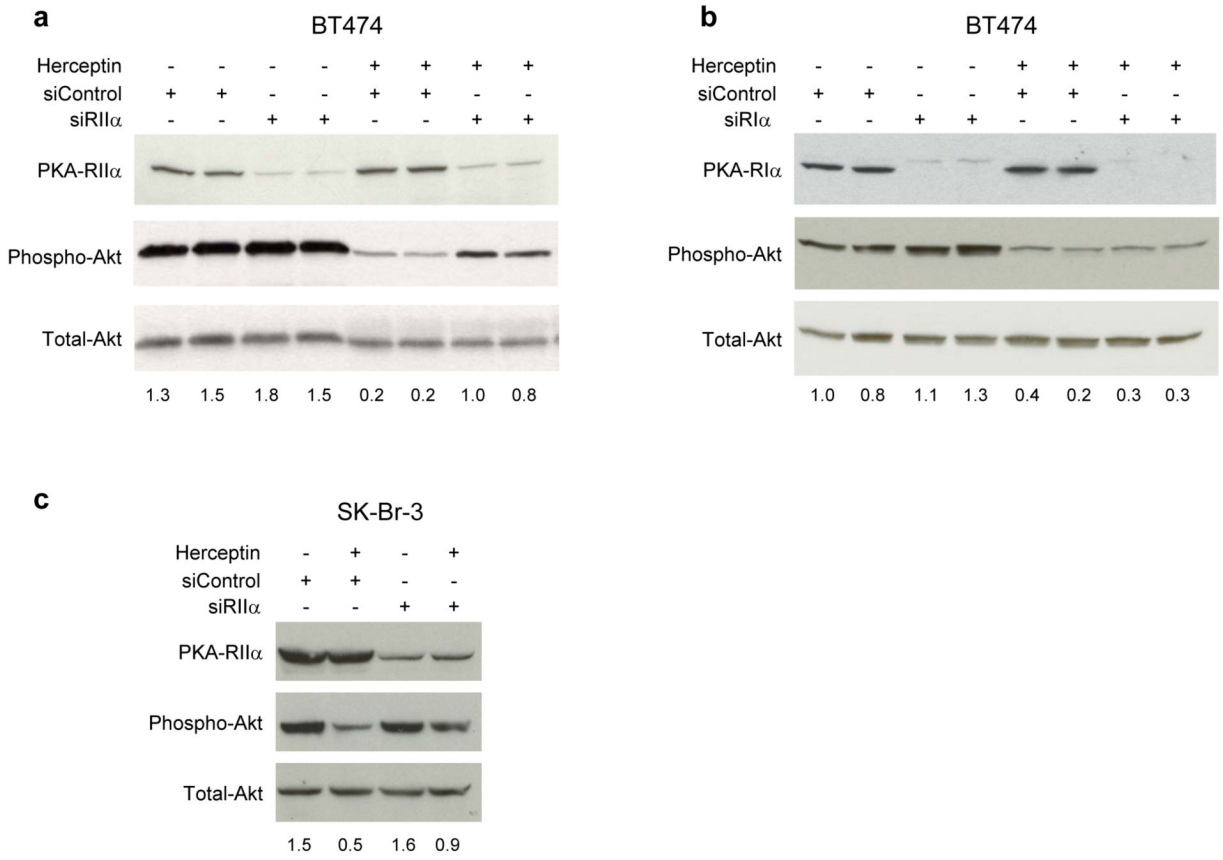

Figure 3.

The effect of PKA-RIIα or PKA-RIα down-regulation on trastuzumab-mediated Akt-dephosphorylation. Parent BT474 cells were transfected with a siRNA targeting (a) PKA-RIIα (siRIIα) or (b) PKA-RIa (siRIα). (c) SK-Br-3 cells were transfected with a siRNA targeting PKA-RIIα (siRIIα). Cells transfected with a non-targeting siRNA (siControl) were used as control. Seventy-two hours post-transfection, cells were incubated for two hours with or without 1.0 μM trastuzumab and then total cellular proteins were extracted. PKA-RIIα (a and c) or PKA-RIα (b) levels and the levels of total Akt and phospho-Akt were analyzed by Western hybridization. The phospho-Akt and total Akt protein levels were quantified by measuring the densities of individual bands on the digitized image of the exposed film. The number under each lane indicates the ratio of phospho-Akt density to total Akt density in that lane.

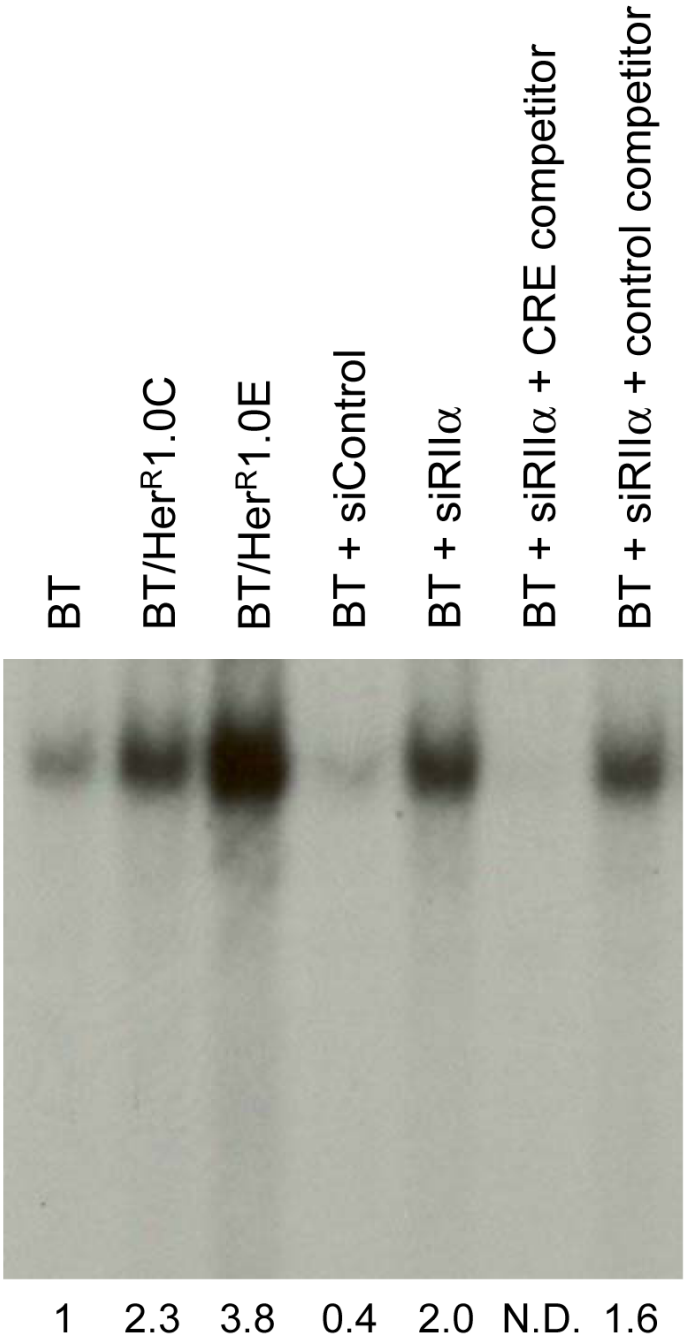

Figure 4.

The effect of PKA-RIIα down-regulation on CRE binding activity. Nuclear extracts were prepared from parent BT474 cells (BT), BT/Her^{R}1.0C and BT/Her^{R}1.0E clones, as indicated, and from BT cells transfected with a non-targeting control siRNA (siControl) or a siRNA targeting PKA-RIIα (siRIIα). Nuclear extracts (5 μg) were subjected to EMSA using a ^{32}P-labeled oligonucleotide probe containing a CRE (Promega) in the absence of unlabelled competitor oligonucleotide or in the presence of 1 pmol of an unlabelled CRE or non-specific (control) competitor oligonucleotide, as indicated. 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.

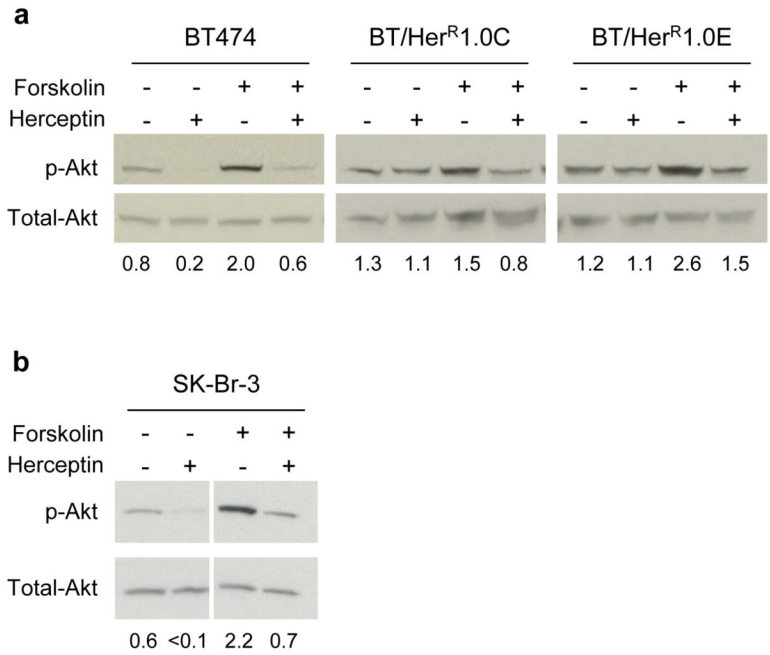

Figure 5.

The effect of PKA activation on the PI3K/Akt pathway. (a) BT474, BT/Her^R and (b) SK-Br-3 cells were incubated with 10 μM forskolin (+) or DMSO (-) for 30 minutes and were then incubated in the presence of trastuzumab (+) or PBS (-) added to the growth medium for an additional two hours. Total cellular proteins were extracted and total Akt and phospho-Akt levels were analyzed by Western hybridization. The number under each lane indicates the density of the phospho-Akt band normalized to that of the total Akt band in the same lane. The panels in (b) both come from different ends of the same gel, with intervening lanes deleted.

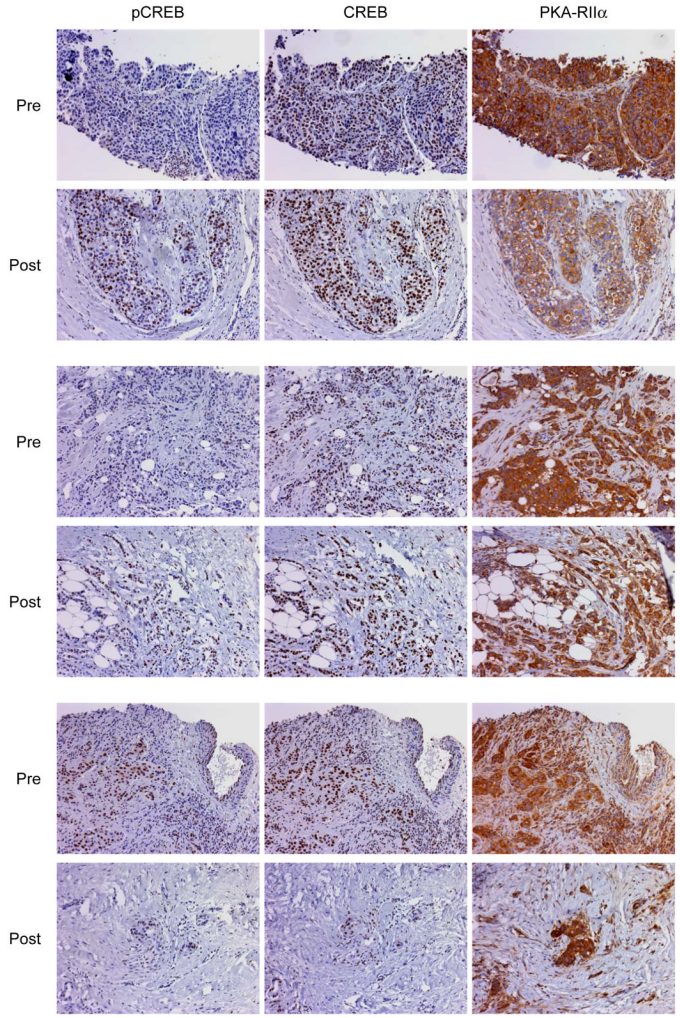

Figure 6.

Immunohistochemistry of clinical samples. Shown are serial sections made from pre-treatment 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 pCREB (left panel), total CREB (middle panel), or PKA-RIIα (right panel), as described in Materials and Methods. The top set of panels is a case in which pCREB staining was stronger and PKA-RIIα staining was weaker after treatment, compared to the corresponding pre-treatment sample; the middle set is a case in which pCREB was stronger but PKA-RIIα was about the same after treatment; and the bottom set is a case in which pCREB was the same or weaker after treatment (see text). Slides were visualized on an Olympus AX70 microscope under a 10X objective lens. Images were digitized using a Retiga EX color camera from Qimaging (Surrey, BC, Canada) and Image Pro Plus imaging software, version 6.3, from Media Cybernetics (Bethesda, MD).

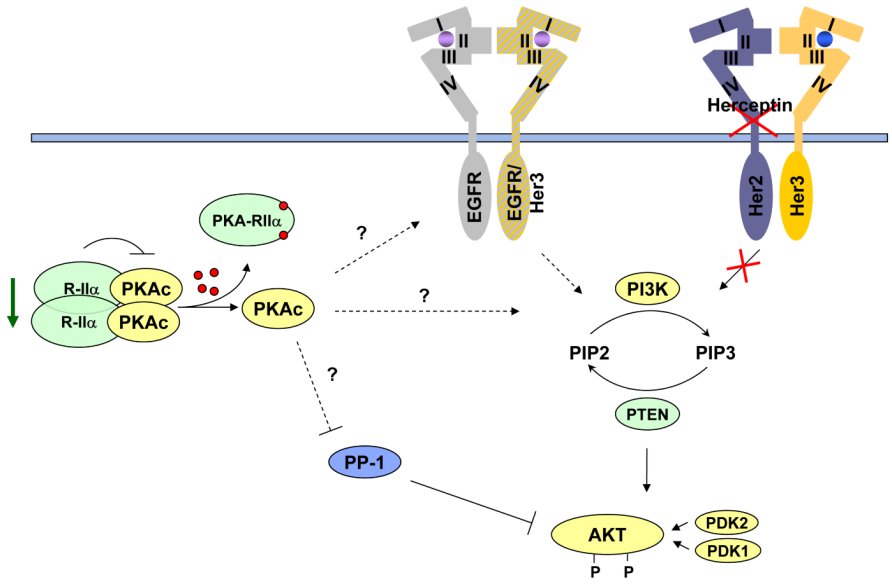

Figure 7.

A working model of PKA's role 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. Release of the RIIα subunit by cAMP (red circles) is illustrated at the left of the figure. The green arrow indicates the down-regulation of PKA-RIIα in trastuzumab-resistant cell lines, also postulated to activate PKAc. The righthand portion of the figure illustrates 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 indicate hypothetical pathways that might be impacted by activated PKA in BT/Her^R cells to mediate sustained PI3K/Akt signaling and trastuzumab resistance. These include direct activation of EGFR or PI3K and indirect activation of pAkt by inhibition of PP-1 (see text). The activation of PI3K/Akt via EGFR (either as a homodimer or as a heterodimer with Her3) upon trastuzumab-mediated inhibition of Her2 is also hypothetical, but consistent with our earlier studies of BT/Her^R cells (39).

Table 1

Log2 ratios for PKA-related genes altered in BT/Her1.0 cells

Symbol	UniGene	Log2 Ratios		
		BT/Her^{R}1.0C vs. BT474	BT/Her^{R}1.0E vs. BT474	
PRKAR2A	Hs.5178	-3.8914	-1.3068	
PRKAR2B	Hs.4330	-1.3888	-1.0042	>1
PPP1R1B	Hs.2861	3.0847	5.8337	>0.4, <1
PPP1R3C	Hs.3030	-2.0406	-1.6536	>0.4, <0.4
PKIG	Hs.4728	-2.0676	-1.0649	<-1, <-0.4
PRKAR1A	Hs.2803	0.1851	0.1831	<-1
PRKAR1B	Hs.5208	-0.0752	-0.0508	
PRKACA	Hs.6316	0.0121	0.0484	
PRKACB	Hs.4873	-0.2845	0.0131	
PRKACG	Hs.1580	-0.0657	0.0604	