t-Darpp stimulates protein kinase A activity by forming a complex with its RI regulatory subunit

Dirk Theile\textsuperscript{a,b,1}, Shuhui Geng\textsuperscript{a,1}, Erin C. Denny\textsuperscript{a,2}, Jamil Momand\textsuperscript{c}, Susan E. Kane\textsuperscript{a,⁎}

\textsuperscript{a} Department of Cancer Biology, City of Hope, 1500 East Duarte Road, Duarte, CA 91010, USA
\textsuperscript{b} Department of Clinical Pharmacology and Pharmacoepidemiology, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany
\textsuperscript{c} Department of Chemistry and Biochemistry, California State University Los Angeles, 5151 State University Drive, Los Angeles, CA 90032, USA

\textbf{ARTICLE INFO}

Keywords:
Breast cancer
Protein kinase A
Regulatory subunit RI
\textit{t-Darpp}

\textbf{ABSTRACT}

t-Darpp is the truncated form of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa (Darpp-32) and has been demonstrated to confer resistance to trastuzumab, a Her2-targeted anticancer agent, via sustained signaling through the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathway and activation of protein kinase A (PKA). The mechanism of \textit{t-Darpp}-mediated PKA activation is poorly understood. In the PKA holoenzyme, when the catalytic subunits are bound to regulatory subunits RI or RII, kinase activity is inhibited. We investigated PKA activity and holoenzyme composition in cell lines overexpressing \textit{t-Darpp} (SK.tDp) or a T39A phosphorylation mutant (SK.tDpT39A), as well as an empty vector control cell line (SK.empty). We also evaluated protein-protein interactions between \textit{t-Darpp} and PKA catalytic (PKAc) or regulatory subunits RI and RII in those cell lines. SK.tDp cells had elevated PKA activity and showed diminished association of RI with PKAc, whereas SK.tDpT39A cells did not have these properties. Moreover, wild type \textit{t-Darpp} associates with RI. Concurrent expression of Darpp-32 reversed \textit{t-Darpp}'s effects on PKA holoenzyme state, consistent with earlier observations that Darpp-32 reverses \textit{t-Darpp}'s activation of PKA. Together, \textit{t-Darpp} phosphorylation at T39 seems to be crucial for \textit{t-Darpp}-mediated PKA activation and this activation appears to occur through an association with RI and sequestering of RI away from PKAc. The \textit{t-Darpp}-RI interaction could be a druggable target to reduce PKA activity in drug-resistant cancer.

1. Introduction

The Her2 (\textit{erbB2/neu}) oncogene is overexpressed in 20–25\% of invasive breast cancers and its expression levels correlate with prognosis, making this EGFR family member an ideal target for stratified breast cancer therapy [1,2]. Trastuzumab is a humanized monoclonal antibody that inhibits Her2-mediated anti-apoptotic and pro-survival signaling through the PI3K/Akt axis [3]. Its clinical application has dramatically improved breast cancer outcomes, but response rates to trastuzumab monotherapy are only 35\% and clinical benefit is estimated at 48\% in patients with Her2 overexpression [4]. Combining trastuzumab with cytotoxic chemotherapy is associated with a longer time to disease progression, a higher rate and longer duration of response, longer survival and reduced risk of death, but this has only been observed during a follow-up of 30 months. Together, the data indicate that there is intrinsic or acquired resistance to trastuzumab in the majority of patients [5,6].

The precise mechanisms of resistance are not completely proven, but sustained PI3K/Akt signaling despite Her2 blockage seems to be crucial [7]. This sustained signaling can be accomplished by different cellular mechanisms, including inhibition of receptor-antibody interaction [8]; gain-of-function mutations in the catalytic subunit of PI3K [9] or down-regulation or loss-of-function mutations in phosphatases such as PTEN [10]; or signaling through alternative receptor tyrosine kinases [11]. PKA activation can also mediate trastuzumab resistance [12,13] and several genes involved in PKA regulation are differentially expressed in cells selected for trastuzumab resistance [12]. One of them is \textit{PPP1R1B}, which encodes Darpp-32 and also an amino-truncated
isoform called t-Darpp [12,14] (Fig. 1). t-Darpp is overexpressed in several cancers, including breast cancer [14,15], and it confers trastuzumab resistance through activation of PI3K/Akt signaling [16–18]. Cells that overexpress t-Darpp also have elevated PKA activity, thus potentially linking the resistance phenotype associated with PKA to the resistance phenotype mediated by t-Darpp [16]. High levels of Darpp-32 reverse t-Darpp’s effects on trastuzumab resistance and PKA activity [16].

The molecular mechanism of t-Darpp-mediated effects on PKA activity is not known, but it most likely functions through direct protein-protein interactions, as does Darpp-32 [19]. Both proteins are regulated by phosphorylation. Phosphorylation at the T75 site in Darpp-32 converts it into a PKA inhibitor [20], whereas phosphorylation at T39, the analogous site in t-Darpp (see Fig. 1), is required for trastuzumab resistance and for activation of the PI3K/Akt pathway [17,21]. The role for T39 phosphorylation in t-Darpp-mediated PKA activation has not been previously reported.

PKA enzymatic activity is primarily controlled by regulatory subunits (RI or RII) that form a holoenzyme complex with the catalytic subunit (PKAc) and inhibit its activity until they are released from the complex by cAMP binding [22]. In this report, we sought to determine if the T39 phosphorylation site is involved in t-Darpp’s activating effect on PKA [16,20], could be mediated by direct interactions with one or more of the PKA holoenzyme subunits. Our findings demonstrate a physical mechanism by which t-Darpp overexpression stimulates PKA activity in cancer cells.

2. Material and methods

2.1. Cell models

The SK-BR-3 human breast cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). Stably transfected SK-BR-3 cells expressing pcDNA3 empty vector (SK.empty), t-Darpp (SK.tDp) or both t-Darpp and Darpp-32 (SK.dDp) were described previously [16]. SK-Br-3 cells transfected with a phosphorylation mutant of t-Darpp in which T39 was mutated to an alanine (SK.tDpT39A) were also previously [16]. SK-Br-3 cells transfected with a phosphorylation mutant of (SK.tDp) or both t-Darpp and Darpp-32 (SK.dDp) were described previously [16].

2.2. PKA activity assays

Two different assays were used to assess PKA activity. The first was a CREB DNA binding assay using the CREB (Phospho-Serine 133) Transcription Factor Assay Kit (Cayman Chemical #10009846, Ann Arbor, MI). In brief, nuclear extracts from 1.5 × 10^6 cells were collected using the Nuclear Extraction Kit (Cayman Chemical #10009846). Nuclear extracts were incubated in a 96-well plate coated with a specific CRE consensus sequence. The activated CRE-CRE complex was detected by the addition of a phospho-Ser133 CREB antibody and a secondary horseradish peroxidase-conjugated antibody. Absorbance measurements at 655 nm (to monitor the signal increase) were obtained on a Molecular Devices microplate reader at 5 min intervals after addition of the Transcription Factor Developing Solution at room temperature. At a reading of 0.4–0.5, the Stop Solution was added, leading to a yellow color change. The final absorbance was measured at 450 nm on the microplate reader. Assays were performed in triplicate and repeated with extracts from three independent experiments.

The second assay was a PKA substrate phosphorylation assay using the FRET-based AKAR4 reporter, kindly provided by J. Zhang (University of California, San Diego). AKAR4 consists of an enhanced yellow fluorescent protein kinase A (PKA) reporter domain and YFP domain separated by a PKA substrate domain. When PKA phosphorylates the substrate domain, a conformation change in the reporter brings the CFP and YFP domains together, leading to a FRET signal [23]. Cells were transiently transfected with 4 μg of the plasmid encoding the AKAR4 reporter using Lipofectamine 2000 (Life Technologies) in appropriate media without antibiotics. After 48 h, cells were washed with PBS (Mediatech Inc., cat. no. 21-030-CV) and fixed using 4% v/v paraformaldehyde. The CFP and YFP channels were excited at 458 nm and 514 nm, respectively. The FRET channel was excited using CFP (458 nm) and captured using the emission range of YFP (535–590 nm). Images were obtained using a Zeiss LSM510 Meta with AIM4.2 LSM imaging software. Relative FRET levels were calculated using the device-implemented nFRET macro 4.2 and normalized to the level present in SK.empty cells.
2.3. Proximity ligation assay

A Duolink proximity ligation assay (Sigma-Aldrich) was used to assess PKA holoenzyme composition and protein-protein interactions in live cells. In brief, 5000 cells/well (triplicate wells for each experiment condition) were seeded in black, clear-bottom 96-well plates (Corning #3340) and incubated for 24 h at 37 °C. After washing with PBS, fixing with 4% paraformaldehyde, permeabilizing with 0.2% v/v Tween-20 in PBS, washing again with 0.05% v/v Tween-20 in PBS (all at room temperature) and blocking (solution contained in the kit) for 1 h at 37 °C, cells were exposed to the primary antibodies (diluted 1:1000 in antibody dilution solution contained in the kit) at 4 °C overnight. The following antibodies were used: mouse anti-RI generated by using mouse RIβ as the immunogen and that recognizes both RIα and RIβ subunits (BD Bioscience #610165), mouse anti-RIP2 (BD Bioscience #612242), rabbit anti-PKAc (Cell Signaling #4782), mouse anti-PKAc (BD Bioscience #610980), rabbit anti-Darpp-32 that recognizes Darpp-32 only (Cell Signaling #2302), and rabbit anti-Darpp that recognizes both t-Darpp and Darpp-32 (Cell Signaling #2306). After primary antibody incubations, cells were washed with wash buffer A (contained in the kit) and exposed to secondary antibodies (PLUS and MINUS probes targeting mouse and rabbit antibodies, respectively, conjugated with oligonucleotides) at room temperature. After 1 h exposure to antibodies, cells were re-washed with wash buffer A and incubated for 1 h at room temperature with the ligation solution (diluted 1:40 in dilution solution) that contained a DNA ligase and oligonucleotides that hybridized to the probes to generate a closed circle if the two probes were in close proximity (< 40 nm). Cells were washed with wash buffer A and exposed to the amplification solution (polymerase dilute 1:80 in dilution solution) for 100 min at 37 °C to begin rolling circle amplification. During this step, fluorescently labeled oligonucleotides were incorporated into the amplification product. Finally, cells were washed with wash buffer B and mounted with mounting media containing DAPI for nuclear counterstaining. Fluorescence (DiRed filter and DAPI filter) was recorded with a Zeiss Z1 Observer microscope and original, non-compressed gray scale pictures (40× magnification) were taken and analyzed with ImageJ. The corrected integrated cell fluorescence (CICF) of a cell was calculated according to the following formula: CICF = integrated cell fluorescence of the cell – (area of the cell × mean fluorescence of the background reading). The CICF value for each cell was normalized to the average CICF from SK.empty cells in the entire experiment and then the normalized CICF values in each well of the experiment were averaged. The average normalized CICF values from three independent wells were averaged to give the final CICF values (± S.D.) represented in the figures. To prevent bias from outliers (very low fluorescence or extremely high fluorescence values from artifact staining), the highest and lowest CICF values in each well were excluded from the CICF calculations. The number of cells analyzed of each cell line was 40–50 in each experiment and each experiment was repeated a minimum of one time.

2.4. Co-immunoprecipitation and Western analysis

Cell lysates from SK.empty, SK.tDp, SK.tDpT39A and SK.dDp cells were prepared in lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific). For immunoprecipitation, each cell lysate (500 μg total protein) was pre-cleared by incubating with 20 μl protein G-agarose beads (Cell Signaling) at 4 °C for 1 h. The pre-cleared lysates were incubated with a mouse monoclonal anti-RIα/β antibody (BD Biosciences #610165) or a rabbit monoclonal anti-Darpp antibody (Cell Signaling #2306), overnight with gentle shaking at 4 °C. A 20 μl aliquot of protein G-agarose beads was added and incubated at 4 °C for 4 h. The beads were pelleted and washed three times with wash buffer (50 mM Tris, 0.1% Nonidet P-40, pH 8.0) and proteins were eluted in Laemmli sample buffer (125 mM Tris-HCl (pH 6.8), 5% SDS, 20% glycerol, 0.2% bromphenol blue) containing 1% (v/v) β-mercaptoethanol by boiling for 5 min. The proteins were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane (GE Healthcare Life Sciences), and analyzed by Western hybridization with anti-Darpp antibody (#2306) or anti-RIα/β antibody (#610165), accordingly. As a negative control, the identical pre-cleared cell lysates were incubated with beads without the precipitation antibody. To confirm the specificity of co-immunoprecipitation, membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) and re-probed for RIα using rabbit monoclonal antibody (Cell Signaling #5675) or t-Darpp + Darpp-32 using rabbit anti-Darpp antibody (#2306).

The same co-immunoprecipitation procedure was used to detect possible co-complexes of PKAc with t-Darpp/Darpp-32 and protein phosphatase-1 (PP1). Rabbit polyclonal antibody recognizing both t-Darpp and Darpp-32 and mouse monoclonal anti-PKAc antibody (BD Biosciences #610980) were used for the immunoprecipitations; the same rabbit anti-Darpp and mouse anti-PKAc antibodies as well as rabbit polyclonal anti-PP1α antibody (Santa Cruz Biotechnology #sc-443) were used for the Western hybridizations. The rabbit polyclonal anti-Darpp antibody was generated against recombinant His-tagged human t-Darpp [24] and affinity-purified on a t-Darpp-conjugated column (ThermoFisher Pierce Custom Services). The antibody concentration was 0.8 mg/ml.

2.5. Statistical analysis

Data were analyzed using GraphPad Prism Version 6.01 (GraphPad Software, San Diego, USA). Differences in CREB CRE complex formation, nFRET levels or CICF values were evaluated by one-way ANOVA corrected for multiple comparisons using Tukey’s post hoc test.

3. Results

3.1. Comparison of protein expression in four stably transfected SK-Br-3 cell clones

Fig. 2 shows the results of Western analysis to quantify relevant protein expression levels in the cell lines used in the current work. There was no detectable Darpp-32 or t-Darpp expression in SK.empty cells, whereas SK.tDp and SK.tDpT39A cells respectively overexpress exogenous wild type t-Darpp or the T39A mutant, respectively, at similar levels. SK.dDp cells co-express high levels of Darpp-32 and t-Darpp. t-Darpp was phosphorylated at residue T39 to the same extent that Darpp-32 was phosphorylated at the corresponding residue T75 (Fig. 2, left panel). RIα, RIβ, and PKAc were at virtually the same levels in all cell clones; RIβ was elevated and RIβ was lower in SK.dDp cells, compared with other three cell lines (Fig. 2, middle and right panels).

3.2. T39 phosphorylation is required for t-Darpp to enhance PKA activity

We have previously reported that cells overexpressing t-Darpp have elevated CREB DNA binding activity (as determined by electrophoretic mobility shift assay), an indirect measure of PKA activity [16]. To confirm this observation and determine if T39 phosphorylation on t-Darpp was required for the phenotype, we used two different assays for PKA activity. In a plate-based CREB DNA binding assay, nuclear extracts from SK.tDp cells (which overexpress t-Darpp) had three-fold higher CREB DNA binding activity than SK.empty cells (which carry an exogenous wild type t-Darpp) or the T39A mutant, respectively, at similar assays for PKA activity. In a plate-based CREB DNA binding assay, nuclear extracts from SK.tDp cells (which overexpress t-Darpp) had three-fold higher CREB DNA binding activity than SK.empty cells (which carry an exogenous wild type t-Darpp) or the T39A mutant, respectively, at similar levels. SK.dDp cells co-express high levels of Darpp-32 and t-Darpp. t-Darpp was phosphorylated at residue T39 to the same extent that Darpp-32 was phosphorylated at the corresponding residue T75 (Fig. 2, left panel). RIα, RIβ, and PKAc were at virtually the same levels in all cell clones; RIβ was elevated and RIβ was lower in SK.dDp cells, compared with other three cell lines (Fig. 2, middle and right panels).

H89 (PKA
inhibitor) and forskolin (PKA activator) were used as controls to confirm functionality of the assay (Supplemental Fig. 1).

3.3. t-Darpp overexpression disrupts PKA holoenzyme composition

Since PKA activity is primarily controlled by its holoenzyme composition or state, we used the Duolink proximity ligation assay to assess spatial proximity of PKAc to its regulatory subunits, RI or RII, in SK.empty, SK.tDp, and SK.tDpT39A cells. For the RI interaction, we used an antibody that detects both the α and β subunits of RI. We detected bright red fluorescence (indicating proximity within 40 nm) in SK.empty and SK.tDpT39A cells but not in SK.tDp cells (Fig. 4A). CICF, a measure of the fluorescence signal intensity, was diminished by 76% in SK.tDp cells compared to SK.empty cells ($p \leq 0.001$), indicating PKAc-RI$\alpha$/β dissociation upon overexpression of t-Darpp. CICF in SK.tDpT39A cells was also lower than in SK.empty cells ($p \leq 0.05$), but was still significantly higher ($p \leq 0.05$) than in SK.tDp cells (Fig. 4B).

We also explored potential alterations in PKAc-RII interactions in these cell lines, using an antibody that detects the α subunit of RII. The PKAc-RII$\alpha$ interaction was slightly increased in SK.tDp cells (36% higher; $p \leq 0.05$) and reduced in SK.tDpT39A cells (67% lower; $p \leq 0.05$), relative to SK.empty cells (Supplemental Fig. 2A). The data suggest that t-Darpp overexpression causes an alteration in the PKA holoenzyme composition, predominantly a decrease in PKAc-RI$\alpha$/β and a small increase in PKAc-RII$\alpha$. The t-Darpp T39A mutant does not appear to decrease PKAc-RI$\alpha$/β significantly but it may decrease PKAc-RII$\alpha$.

Darpp-32 is known to reverse t-Darpp’s effect on PKA-mediated CREB DNA binding activity ([16]). Thus, we evaluated the PKAc-RI$\alpha$/β interaction in SK.dDp cells that overexpress both t-Darpp and Darpp-32. We found that SK.dDp cells had significantly higher levels of PKAc-RI$\alpha$/β interaction than SK.tDp cells and SK.tDp T39A cells ($p \leq 0.05$)
essentially the same as the interaction seen in SK.empty cells (Fig. 4A, B). This suggests that Darpp-32 overexpression reverses t-Darpp’s effect on the PKAc-Rla/β holoenzyme composition.

3.4. t-Darpp interacts directly with RI regulatory subunit

To investigate further the molecular reasons for enhanced PKA activity and altered PKA holoenzyme composition in SK.tDp cells, we used the proximity ligation assay to evaluate t-Darpp protein-protein interaction with PKA subunits RI, RII or PKAc itself. t-Darpp interaction with the proximity ligation assay to evaluate t-Darpp protein-protein interactivity and altered PKA holoenzyme composition in SK.tDp cells, we used

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In SK.tDp T39A cells, the t-Darpp-Rla/β interaction was significantly diminished compared to SK.tDp cells (p ≤ 0.05), but was still slightly increased (3-fold; p ≤ 0.05) compared to SK.empty cells (Fig. 5A). In addition, a single proximity ligation experiment showed a concentration-dependent loss of t-Darpp-Rla/β interaction and concomitant increase in the PKAc-Rla/β interaction signal in the presence of small-molecule inhibitors of cdk1 and cdk5, the kinases responsible for T39 phosphorylation on t-Darpp [20,24] (Supplemental Fig. 4).

In a separate experiment, CICF values for the interaction between Rla/β and t-Darpp + Darpp-32 in SK.dDp cells (using an antibody that detects both t-Darpp and Darpp-32 and an antibody that detects Rla/β) were approximately twice the CICF values seen in SK.tDp cells (data not shown). This prompted us to evaluate the specific interaction between Darpp-32 and Rla/β using an antibody to the amino-terminal domain of Darpp-32 that is absent from t-Darpp. The proximity ligation assay revealed CICF values that were about 4-fold higher in SK.dDp cells than SK.empty and SK.tDp cells (Fig. 5B), suggesting that Darpp-32 can also bind the Rla/β regulatory subunit.

To evaluate the suspected interaction between t-Darpp and Rla/β and to determine the relevance of the T39A mutation using another experimental approach, we conducted co-immunoprecipitation assays followed by Western analysis using lysates from SK-empty, SK.tDp, SK.tDp T39A, and SK.dDp cells. Immunoprecipitation with an antibody against Rla/β pulled down t-Darpp from SK.tDp lysates and to a much lesser degree from SK.tDp T39A lysates (Fig. 6A). The reciprocal approach, immunoprecipitation with anti-Darpp antibody followed by Western detection of Rla/β, confirmed the interaction between t-Darpp and Rla/β (Fig. 6B). The Rla/β immunoprecipitation and reciprocal
Darpp immunoprecipitation further substantiated the interaction between Rla/β and both t-Darpp and Darp-32 when lysates from SK.dDp cells were analyzed, with Darp-32 perhaps interacting with Rla/β to a greater extent even than t-Darpp (Fig. 6C).

3.5. t-Darpp/Darpp-32 and PP1 are not in the complex with PKAc

We also wanted to use co-immunoprecipitation to confirm the lack of a direct interaction between Rla/β and both t-Darpp and Darp-32 when lysates from SK.dDp cells were analyzed, with Darp-32 perhaps interacting with Rla/β to a greater extent even than t-Darpp (Fig. 6C).

4. Discussion

Sustained PI3K/Akt signaling is considered a hallmark of trastuzumab resistance and enhanced PKA activity is reported as one of the mechanisms contributing to trastuzumab resistance and possibly Akt activation [12,13,16,25]. Both pharmacological (e.g. forskolin treatment) and knock-down approaches to influence PKA holoenzyme composition are sufficient to alter PKA enzymatic activity, Akt phosphorylation state and trastuzumab resistance [12]. Since t-Darpp confers trastuzumab resistance and PKA activation [16–18], we wanted to know if t-Darpp could affect PKA holoenzyme composition as a possible mechanism of PKA activation.

Darp-32 is an effector of PKA's inhibitory effect on PP1 and it is a feedback inhibitor of PKA itself [19,20]. Darp-32 inhibits PP1 via a protein-protein interaction that requires the N-terminal domain that is absent from t-Darpp, but the molecular mechanism by which Darp-32 inhibits PKA is not known. We focused on the T39 phosphorylation site in t-Darpp because phosphorylation at this site is required for t-Darpp's ability to increase trastuzumab resistance and PI3K/Akt signaling.
mutant co-precipitated with RIα/β interaction by 5-fold (Fig. 5A). Likewise, a very low level of the T39A PKAc-RIα interaction compared to cells transfected with an empty vector (Fig. 4). The T39A mutant with RIα/β phosphorylation at the analogous T75 site in Darpp-32 and PP1 are absent from the PKAc complex. (A) Lysates from SK.empty, SK.dDp, SK.dDpT39A, and SK.dDp cells were subjected to immunoprecipitation with polyclonal rabbit anti-Darpp antibody followed by Western analysis using mouse anti-PKAc antibody (BD Biosciences #610980) (upper panels) or rabbit anti-PP1α antibody (Santa Cruz Biotechnology #sc-443) (middle panels). Stripping and re-probing for t-Darpp/Darpp-32 is shown in the bottom panels. (B) Lysates were immunoprecipitated with mouse anti-PKAc antibody (#610980) followed by Western analysis using polyclonal rabbit anti-Darpp antibody or rabbit anti-PP1α antibody (sc-443). Stripping and re-probing for PKAc for each experiment is shown in the bottom panels.

Consistent with our previous report [16], t-Darpp overexpression enhanced PKA activity as determined by CREB DNA binding and PKA substrate phosphorylation assays (Fig. 3). Concurrently, t-Darpp overexpression led to a dissociation of PKAc from its RI regulatory subunit (Fig. 4) and it appears that this dissociation was mediated by direct binding of t-Darpp to Ria/β (Figs. 5, 6), which sequestered Ria/β away from PKAc. Such an event would be consistent with canonical activation of PKA, similar to what is seen when CAMP binds Ria/β and causes dissociation and activation of PKA. A protein-protein interaction-based mechanism of PKA activation has already been described for p90 ribosomal kinase 1, which also binds RI subunits and subsequently enhances the enzymatic activity of the released PKAc [26]. Our new data suggests the same mode of action for t-Darpp.

Cells expressing the T39A mutant of t-Darpp had only modestly elevated PKA activity (Fig. 3) and exhibited only slightly diminished PKAc-Ria/β interaction compared to cells transfected with an empty vector (Fig. 4). Comparing the t-Darpp-Ria/β interaction in the different cell lines using proximity ligation assays, the signal from the wild-type t-Darpp interaction exceeded that from the T39A mutant interaction by 5-fold (Fig. 5A). Likewise, a very low level of the T39A mutant co-precipitated with Ria/β in SK.dDpT39A cells (Fig. 6A). This is consistent with proximity ligation assays showing a weak interaction of the T39A mutant with Ria/β in SK.dDpT39A cells (Fig. 5A) and a slightly diminished PKAc-Ria/β interaction in SK.dDpT39A cells, compared to the interaction observed in SK.empty cells (Fig. 4). Together, these data suggest an interaction between the T39A mutant protein and Ria/β, but such interaction might be too weak to translate into significant PKAc-Ria/β disruption (Fig. 4) and subsequent PKA activation (Fig. 3). All of these results suggest that T39 phosphorylation plays an important role in t-Darpp’s capacity to modulate PKA activity. This same site has already been shown to be critical for t-Darpp’s ability to activate EGFR signaling [21], to cause sustained Akt phosphorylation and to confer trastuzumab resistance [17], perhaps suggesting that these outcomes are downstream effects of t-Darpp-mediated PKA stimulation.

It should be noted that the diminished PKAc-Ria/β interaction seen in SK.dDp cells (Fig. 4) was accompanied by a slight increase in the interaction between PKAc and Ria, as determined by proximity ligation assay (Supplemental Fig. 2A). However, this increased PKAc-Ria interaction did not prevent enhanced PKA activity, which suggests that there might not be enough PKA-RiAs holoenzyme in these cells to inhibit PKA activity effectively and the net impact of t-Darpp overexpression would still be PKA activation. This speculation is supported by our previous finding that Ria expression is down-regulated in other trastuzumab-resistant breast cancer cells that have enhanced PKA activity [12], suggesting a need for low Ria levels constitutively with t-Darpp binding to Ria/β to achieve PKA activation. It is also possible that sequestering Ria/β away from PKAc (by t-Darpp) has an effect on PKAc localization in a way that precludes significant Ria binding to PKAc, thus promoting PKA activation [27]. Further analysis of PKA, Ria and Ria subcellular localization is required to determine if enhanced PKA activity is due to altered localization in SK.dDp cells.

We have previously reported that Darpp-32 reverses t-Darpp’s effects on drug resistance, Akt phosphorylation and PKA activity [16]. This antagonistic effect of Darpp-32 was also seen in the proximity ligation assays presented here, in which concurrent expression of Darpp-32 with t-Darpp (in SK.dDp cells) appeared to reverse t-Darpp’s effect on the PKAc-Ria/β interaction. SK.dDp cells exhibited four-fold higher PKAc-Ria/β interaction than SK.dDp cells and the level of this interaction was not significantly different from what was observed in SK.empty cells (Fig. 4B). Interestingly, we did detect association of Darpp-32 with Ria/β in SK.dDp cells when we used a Darpp-32-specific antibody in the proximity ligation assay (Fig. 5B) and Darpp-32-Ria/β association was detected in co-immunoprecipitation assays (Fig. 6C). The interaction might be relatively weak, given the high levels of Darpp-32 in SK.dDp cells (Fig. 2) and the relatively lower interaction with Ria/β in the proximity ligation assay (4-fold over background vs. SK.empty).
the 14-fold interaction seen for t-Darpp-R1α/β in SK.tDp cells (Fig. 5). The co-immunoprecipitation data, on the other hand, suggest a stronger pull-down of Darpp-32 than t-Darpp in the R1α/β immunoprecipitation (Fig. 6C).

Taken together, the data on PKA holoenzyme state (PKAc-R1α/β interaction) and PKA activity, together with published reports of Darpp-32 as a PKA inhibitor [20] and our own studies of t-Darpp and Darpp-32 [16] support a model in which Darpp-32 and t-Darpp are antagonizing proteins whose interactions with R1α/β produce very different effects on PKA holoenzyme and PKA activity (see Fig. 8). The molecular basis for this antagonism is unclear, but one possibility is that the amino-terminal domain unique to Darpp-32 (amino acids 1–36) plays a role, either by directly preventing PKAc-R1α/β dissociation (perhaps even by “masking” the T39 site that is crucial for PKA activation and PKAc-R1α/β dissociation mediated by t-Darpp) or by an indirect effect on t-Darpp and/or R1α/β localization or activity. We also observed that R1β is elevated and R1β is lower in cells expressing t-Darpp and Darpp-32 compared to our other three cell lines (see Fig. 2). The mechanism for this is unclear, but it is possible that the effect of the different subunit levels is to influence PKA holoenzyme composition and thus make PKA refractory to CREB activation. Indeed, R1β is associated with mitochondrial membranes and R1β is found in the soluble fraction of cells [28], so perhaps the altered levels of these competing PKA holoenzyme components cause R1β to sequester PKAc to the mitochondria, thus preventing access of PKAc to CREB and other soluble substrates. Regardless of the molecular mechanism, the net effect in the presence of Darpp-32 is sustained PKAc-R1α/β interaction and resulting PKA inhibition, a known role for Darpp-32 in the PKA signaling pathway [19].

Given the opposite effects of t-Darpp and Darpp-32 in this pathway and on the PKA holoenzyme, it seems reasonable to suggest that the ratio of t-Darpp and Darpp-32 proteins should influence the PKA signaling phenotype of cancer cells and eventually the growth/survival phenotype. Small molecule drugs that inhibit t-Darpp interaction with R1α/β or perturb the t-Darpp:Darpp-32 ratio (towards high Darpp-32 and low t-Darpp) could have a positive impact on tumor growth and drug resistance.

5. Conclusions

We present here a model explaining part of the trastuzumab-resistant phenotype of breast cancer cells that overexpress t-Darpp. Our model is that t-Darpp sequesters regulatory subunit R1α/β away from PKAc, leading to enhanced PKA activity and sustained PI3K/Akt signaling. t-Darpp phosphorylation at the T39 residue promotes its complex formation with R1α/β, thus enhancing its effect on PKA activity. The t-Darpp-R1α/β interaction could be a druggable target to reduce PKA activity and Akt signaling in drug-resistant cancer.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgments

The authors wish to thank Cécile Donohue for technical assistance and Brian Armstrong and Loren Quintanar from the Light Microscopy Core for their technical and scientific assistance. The authors also acknowledge Patrycja Magdziarz for purification of His-tagged t-Darpp.

Funding

This work was supported by grants from the National Institutes of Health (GM105898, CA33572).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2017.08.012.

References
