Inhibition of ezrin causes PKCα-mediated internalization of erbb2/HER2 tyrosine kinase in breast cancer cells

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Unlike other ErbB family members, HER2 levels are maintained on the cell surface when the receptor is activated, allowing prolonged signaling and contributing to its transforming ability. Interactions between HER2, HSP90, PMCA2, and NHERF1 within specialized plasma membrane domains contribute to the membrane retention of HER2. We hypothesized that the scaffolding protein ezrin, which has been shown to interact with NHERF1, might also help stabilize the HER2–PMCA2–NHERF1 complex at the plasma membrane. Therefore, we examined ezrin expression and its relationship with HER2, NHERF1, and PMCA2 levels in murine and human breast cancers. We also used genetic knockdown and/or pharmacologic inhibition of ezrin, HSP90, NHERF1, PMCA2, and HER2 to examine the functional relationships between these factors and membrane retention of HER2. We found ezrin to be expressed at low levels at the apical surface of normal mammary epithelial cells, but its expression is up-regulated and correlates with HER2 expression in hyperplasia and tumors in murine mammary tumor virus-Neu mice, in human HER2-positive breast cancer cell lines, and in ductal carcinoma in situ and invasive breast cancers from human patients. In breast cancer cells, ezrin co-localizes and interacts with HER2, NHERF1, PMCA2, and HSP90 in specialized membrane domains, and inhibiting ezrin disrupts interactions between HER2, PMCA2, NHERF1, and HSP90, inhibiting HER2 signaling and causing PKCα-mediated internalization and degradation of HER2. Inhibition of ezrin synergizes with lapatinib in a PKCα-dependent fashion to inhibit proliferation and promote apoptosis in HER2-positive breast cancer cells. We conclude that ezrin stabilizes a multiprotein complex that maintains active HER2 at the cell surface.

Breast cancer is the most common cancer and the second most common cause of cancer deaths in United States women. Approximately 25–30% of breast cancers overexpress ErbB2/HER2/Neu (HER2), and transgenic expression of ErbB2 in the mouse mammary gland is sufficient to cause invasive mammary carcinomas. ErbB2/HER2 is a receptor tyrosine kinase that functions as an obligate heterodimer with other ErbB family receptors, especially EGFR (ErbB1/HER1) and ErbB3/HER3 in breast cancer cells. One characteristic that contributes to HER2’s transforming ability is its stability at the plasma membrane. In contrast to other ErbB family members, when activated, HER2 resists internalization and degradation and remains on the cell surface to signal for prolonged periods. Although the mechanisms underlying the retention and/or rapid recycling of HER2 at the cell surface are not fully understood, reports suggest that it must interact with the chaperone HSP90, the plasma membrane calcium ATPase2 (PMCA2), and the scaffolding molecule sodium–hydrogen exchanger regulatory factor 1 (NHERF1) to avoid endocytosis and degradation within the cell.

NHERF1 is a scaffolding protein containing two tandem PDZ domains and a C-terminal ezrin/radixin/moesin/merlin (ERM)–binding domain. It binds to membrane proteins through its PDZ domains, facilitating the formation of multiprotein complexes that are tethered to the actin cytoskeleton through ERM proteins such as ezrin. Tumor NHERF1 levels have been demonstrated to correlate with HER2 expression, HER2 localization, and HER2 signaling. NHERF1 links plasma membrane–signaling complexes to the cortical cytoskeleton through ERM proteins such as ezrin. In the cytoplasm, ezrin is maintained in an inactive confirmation by intramolecular monomeric interactions or head-to-tail dimeric interactions, both of which mask critical

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2 The abbreviations used are: EGFR, epidermal growth factor receptor; ERM, ezrin/radixin/moesin/merlin; MMTV, murine mammary tumor virus; DCIS, ductal carcinoma in situ; PDH, pleckstrin homology; EGF, epidermal growth factor; PLA, proximity ligation assay; IP, immunoprecipitation; shRNA, short hairpin RNA; KD, knockdown; PMA, phorbol 12-myristate 13-acetate; HA, hemagglutinin; CA, constitutively active; BrdU, bromodeoxyuridine; DAPI, 4’,6-diamidino-2-phenylindole.
binding motifs. Ezrin is activated by interactions of its FERM (4.1 protein, ezrin, radixin, moesin) domain with phosphatidylinositol 4,5-bisphosphate (PIP2) at the plasma membrane and by phosphorylation of a conserved threonine residue (Thr-567) in the C-terminal region. When activated, ezrin assumes a configuration that allows binding to actin, NHERF1, and other partner proteins (27–30). Both the overall level of ezrin expression and its pattern of localization in tumor cells predict outcome in patients with breast cancer, and, in particular, several studies have suggested that ezrin levels correlate with HER2 expression (15, 27, 29, 31–34). In this work, we found that ezrin contributes to HER2 membrane retention and downstream signaling by helping to stabilize membrane signaling complexes containing HER2, EGFR, HER3, NHERF1, PMCA2, and HSP90.

Results

Ezrin levels correlate with HER2 levels in breast cancers

We examined the relationships between HER2, NHERF1, and ezrin expression in mRNA microarray data from the METABRIC breast cancer database (35–37). The genes were statistically likely to be co-expressed with each other in breast tumors, as noted by the positive and highly significant log odds ratios (Fig. 1A). In addition, when we examined each pair of mRNA comparisons, we found highly significant positive correlations between ezrin and HER2 or NHERF1 in breast tumors (Fig. 1, B–D). However, the relationships between mRNA expression were modest, as judged by the r values. Compared with immortalized MCF10A human breast epithelial cells, which have low levels of ERBB2 gene expression, the levels of ezrin mRNA were increased in two standard HER2-positive breast cancer cell lines, BT474 and SKBR3, both of which overexpress ErbB2/HER2 (Fig. 1E).

Next, we examined the pattern of ezrin protein expression using immunofluorescence in normal mouse mammary glands and in hyperplastic lesions and tumors from MMTV-Neu transgenic mice, which overexpress WT HER2 in mammary epithelial cells and serve as a standard model of HER2-positive breast cancer (3). In normal mammary ducts, ezrin was located exclusively in the apical plasma membrane of luminal epithelial cells, and HER2 was not detected (Fig. 1F). In hyperplastic

| GeneA | GeneB | p-value | Log odds ratio | Association               |
|-------|-------|---------|----------------|---------------------------|
| Ezrin | HER2  | <0.001  | 0.776          | Tendency toward co-occurrence (significant) |
| Ezrin | NHERF1| <0.001  | 1.268          | Tendency toward co-occurrence (significant) |
| HER2  | NHERF1| <0.001  | 0.806          | Tendency toward co-occurrence (significant) |

Figure 1. A, mutual exclusivity analysis of ERBB2 (HER2), EZR (Ezrin), and SLC9A3R1 (NHERF1) mRNA expression from the METABRIC breast cancer database. B–D, correlations between ezrin (y axis) and HER2 (x axis) (B), NHERF1 (y axis) and HER2 (x axis) (C), and ezrin (y axis) and NHERF1 (x axis) mRNA levels in individual tumors from the METABRIC breast cancer database. E, Ezrin mRNA expression in breast cancer cell lines, as assessed by quantitative PCR. Error bars represent mean ± S.E. for three experiments. ***, p < 0.0005; ****, p < 0.00005. F, typical immunofluorescence staining for HER2 (green) and Ezrin (red) in normal murine virgin mammary ducts (top row), hyperplastic lesions from MMTV-Neu mice (center row), and mammary tumors from MMTV-Neu mice (bottom row). Right panels, merged images with DAPI staining (blue). Yellow arrows, apical plasma membrane; white arrows, basolateral membrane. G, representative HER2 (green) and Ezrin (red) immunofluorescence staining in human HER2-negative (top row) or HER2-positive (bottom row) DCIS lesions. Right panels, merged images with DAPI staining. Yellow arrow, apical plasma membrane; white arrow, basolateral membrane. Scale bars = 10 μm.
lesions and in tumors from MMTV-Neu mice, both HER2 and ezrin were expressed and co-localized at the plasma membrane (Fig. 1F). Furthermore, ezrin was no longer located specifically in the apical membrane but co-localized with HER2 in both basolateral and apical plasma membranes. We also examined ezrin expression in human ductal carcinoma in situ (DCIS). Ezrin immunofluorescence was detected at the apical plasma membrane in HER2-negative DCIS samples (n = 3) (Fig. 1G). In HER2-positive DCIS (n = 6), ezrin immunofluorescence was more prominent and was noted throughout the plasma membrane, where it co-localized with HER2 staining (Fig. 1G). These results demonstrate that HER2-mediated tumor formation alters both overall ezrin expression as well as its polarized membrane localization and that membrane-associated ezrin co-localizes with HER2 in murine and human breast cancer cells.

**Ezrin, NHERF1, and HER2 co-localize at the plasma membrane**

We next examined ezrin immunofluorescence in SKBR3 cells. At baseline, in serum-containing medium, ezrin co-localized with NHERF1 and HER2 in actin-containing plasma membrane domains that protruded from the apical aspect of the cells, as evident from the Z stack reconstructions (Fig. 2A). Although NHERF1 could be detected throughout the cells, it co-localized with ezrin primarily within protruding membrane domains. Ezrin must be phosphorylated at threonine 567 to bind actin and associate with the plasma membrane (27, 28, 30), and phosphorylated Ezrin was almost exclusively seen within membrane protrusions, where it co-localized with HER2 (Fig. 2B). The C terminus of activated ezrin associates with PIP₂, which helps to anchor ezrin to the plasma membrane (27, 29, 30). Therefore, we also examined interactions between PIP₂ and ezrin or HER2. The pleckstrin homology (PH) domain of phospholipase C-δ1 (PLCδ1) binds to PIP₂, allowing us to use GFP-tagged PLCδ-PH (GFP-C1-PLCδ-PH) to examine the distribution of PIP₂ within SKBR3 cells (38). As shown in Fig. 2C, GFP-C1-PLCδ-PH co-localized with both ezrin and HER2 prominently within protruding plasma membrane domains, supporting the idea that activated ezrin localizes to sites of active membrane HER2 signaling. In fact, co-localization of ezrin and HER2 was promoted in SKBR3 cells by treatment with EGF or Neuregulin, which would be expected to activate either EGFR/HER2 or HER3/HER2 heterodimers, respectively. As shown in Fig. 2D, in cells without growth factors in serum-free medium, ezrin distributed more diffusely within and/or near the plasma membrane and did not completely co-localize with HER2 expression. However, after 2 h of treatment with either EGF or NRG1, ezrin immunofluorescence became concentrated within membrane protrusions and co-localized more closely with HER2 (Fig. 2D). We and others have shown that HER2 co-localizes with EGFR and HER3 within membrane protrusions (10, 11), and we also observed co-localization of ezrin with both EGFR and HER3 in cells at baseline in serum-containing medium (Fig. 2E). In contrast to HER2, treatment of SKBR3 cells with either EGF or NRG1 was associated with internalization of EGFR and HER3 (Fig. 2F), and the internalized EGFR or HER3 no longer co-localized with ezrin, suggesting that ezrin remains associated with activated HER2/EGFR or HER2/HER3 heterodimers, which are retained at the cell surface (Fig. 1F) (4, 6). To determine whether HER2 and Ezrin were contained within the same complex, we first exposed cells to a cross-linking reagent, succinimidyl 6-(3-(2-pyridyldithio)propionamido)hexanoate and immunoprecipitated HER2, which also pulled down ezrin (Fig. 2G). In addition, we also performed proximity ligation assays (PLAs) in the presence or absence of EGF to determine whether, like NHERF1, ezrin was recruited to interact with HER2 in response to its activation (11, 39). As shown in Fig. 2H, treatment of SKBR3 cells with EGF for as little as 10 min led to an increase in close interactions between HER2 and NHERF1 and between HER2 and Ezrin. Together, the co-immunofluorescence, co-immunoprecipitation, and PLA data demonstrate that ezrin becomes associated with a multiprotein complex containing activated HER2 and NHERF1.

**Ezrin is required for HER2 signaling and membrane retention**

We used a specific shRNA to knock down ezrin expression in SKBR3 cells, and, compared with control cells (transfected with nonspecific shRNA), EzrinKD cells had reduced levels of ezrin, total HER2, and pHER2 (Tyr-1221/1222) (Fig. 2I). Although the reduction in pHER2 (94%) was greater than the reduction in total HER2 (82%), it was difficult to ascertain whether reduced levels of pHER2 were the result of a specific decrease in HER2 phosphorylation or the overall reduction in total HER2. Therefore, we also treated SKBR3 and BT474 cells with NSC668394, a pharmacological inhibitor of Ezrin that prevents its phosphorylation and activation (40). Compared with knocking down Ezrin, SKBR3 and BT474 cells exposed to 5 μM NSC668394 for 16 h only showed a modest reduction (14% for SKBR3 and 23% for BT474) in total HER2 levels but a much greater reduction (96% for SKBR3 and 76% for BT474) in phospho-HER2 levels (Fig. 2I). These results show that inhibition of ezrin reduced HER2 activation, as assessed by the steady-state levels of pHER2, and that the reductions in pHER2 were not simply due to reductions in total HER2 abundance.

Knocking down ezrin expression or inhibiting ezrin function led to the effacement of the actin-rich membrane protrusions and a redistribution of HER2 throughout the plasma membrane (Fig. 2, K and L). As shown in confocal optical sections (Fig. 2L), a portion of HER2 was also internalized, as evidenced by staining in the cytoplasm of the cells (Fig. 2, L and M). Interestingly, in Ezrin KD cells, the internalized HER2 often appeared to be localized within bleb-like structures, whereas the internalized HER2 appeared more punctate in cells treated with NSC668394 (Fig. 2L). In both instances, knocking down ezrin or inhibiting ezrin function disrupted co-localization between NHERF1 and HER2, and, in the case of NSC668394, the remaining ezrin protein also no longer co-localized with HER2 (Fig. 2L). In contrast to control cells (Fig. 2, E and F), in the Ezrin KD cells, HER2 continued to co-localize with EGFR and HER3, but within bleb-like structures inside the cells (Fig. 2N). Thus, Ezrin contributes to the cell surface retention of activated HER2, and disruption of ezrin expression or function results in the internalization of HER2 and reduction in its activation.
Ezrin stabilizes membrane HER2
**PMCA2 and NHERF1 are necessary for interactions between HER2 and Ezrin**

Ezrin is known to interact with NHERF1, and we have shown that NHERF1, in turn, interacts with the calcium pump PMCA2 to stabilize HER2 at the cell surface (10, 11). Therefore, we examined how stably knocking down either PMCA2 or NHERF1 affected interactions between HER2 and ezrin in SKBR3 cells. In PMCA2KD and NHERF1KD cells, ezrin could be found within the cells in prominent aggregates that no longer co-localized with HER2 (Fig. 3, A and B, top rows). As we described previously, stimulation of PMCA2KD and NHERF1KD cells with EGF leads to abnormal internalization of HER2 (10, 11), which did not co-localize with the internalized ezrin (Fig. 3, A and B, bottom rows). Treatment of control cells with EGF and NRG1 for 2 h induced activation (phosphorylation) of Ezrin (Thr-567), but in PMCA2KD and NHERF1KD cells, phospho-ezrin levels did not increase in response to EGF or NRG1 (Fig. 3, C and D). Prior work suggested that an increase in intracellular calcium concentrations mediated the effects of loss of PMCA2 or NHERF1 on HER2 localization and signaling (10, 11); therefore, we also assessed the effects of raising the intracellular calcium concentration on interactions between ezrin and HER2. Treatment of control cells with 10 mM calcium and ionomycin for 16 h caused internalization of HER2 as well as loss of co-localization between ezrin and HER2 (Fig. 3, E and F). Calcium and ionomycin treatment also reduced the relative co-immunoprecipitation of ezrin with HER2 (Fig. 3G). Together, these data demonstrate that ezrin interacts with HER2 in the presence of PMCA2 and NHERF1 and that the interactions between HER2 and ezrin are sensitive to intracellular calcium concentrations.

**Activation of PKCα causes internalization of HER2**

PKCα is a membrane-associated serine-threonine kinase activated by intracellular calcium and diacylglycerol that has been shown previously to regulate endocytosis of ErbB family members (41–46). Because increased intracellular calcium is associated with internalization of HER2 and activation of PKCα, we next examined whether activation of PKCα might affect interactions between ezrin and HER2 and lead to internalization of HER2. First, we treated SKBR3 cells with two separate pharmacologic PKCα agonists, PMA and bryostatin1. Treatment with these agents for 16 h reduced the relative ability to co-immunoprecipitate ezrin with HER2 (Fig. 4A) and reduced pHER2 but not total HER2 levels (Fig. 4B). PMA and bryostatin1 also both decreased phospho-ezrin levels, although PMA did so to a greater extent (Fig. 4C). Immunofluorescence staining demonstrated that PMA and bryostatin1 caused internalization of ezrin, NHERF1, and HER2 and effacement of membrane protrusions (Fig. 4, D–F). Although immunofluorescence staining for NHERF1 and ezrin remained co-localized within the cells (Fig. 4F), co-localization of HER2 with NHERF1 or ezrin was reduced within the cytoplasm compared with the cell surface (Fig. 4, D and E). Neither PMA nor bryostatin1 are specific PKCα agonists, so we also overexpressed HA-tagged WT PKCα (WT-PKCα) or HA-tagged constitutively active PKCα (CA-PKCα) in SKBR3 cells and performed immunofluorescence staining for the HA tag, HER2, Ezrin, and NHERF1. As shown in Fig. 4F, overexpressed WT-PKCα was found throughout the cytoplasm but also specifically co-localized with HER2, ezrin, and NHERF1 within membrane protrusions. However, overexpression of WT-PKCα did not lead to HER2 internalization or disrupt the membrane protrusions (Fig. 4, I and J). By contrast, cells overexpressing CA-PKCα demonstrated a reduction in membrane protrusions and prominent internalization of HER2, ezrin, and NHERF1 together with CA-PKCα (Fig. 4, I and K). Likewise, when we activated the overexpressed WT-PKCα with PMA or ionomycin and extracellular calcium, we observed a reduction in membrane protrusions and internalization of HER2 and ezrin with WT-PKCα (Fig. 4, L and M). Taken together, these results show that PKCα is found within membrane signaling domains containing HER2, ezrin, and NHERF1 and that, when activated, PKCα promotes the internalization of HER2.

**Figure 2.** A, immunofluorescence staining for NHERF1 and Ezrin (top row), HER2 and Ezrin (center row), and phalloidin (actin) and Ezrin (bottom row) in SKBR3 cells. Right panels, merged staining to highlight co-localization (yellow). For all panels, insets on top and on the right of enlarged images represent Z stacks in two different orientations: the apical side of the cell facing down in the top *inset* and to the left in the *side inset*. Far right panels, enlargements of Z stack reconstructions. White arrows point to co-localizations in membrane protrusions. B, immunofluorescence staining of HER2 and phospho-Ezrin in SKBR3 cells. White arrows point to co-localization in membrane protrusions. C, co-immunofluorescence staining for Ezrin (top row) and HER2 (bottom row) in SKBR3 cells expressing PH-PLC- GFP. White arrows point to co-localization in apical membrane protrusions. D, immunofluorescence staining for HER2 and ezrin in SKBR3 cells in serum-free medium (top row) and after 2 h of treatment with EGF (center row) or NRG1 (bottom row). Right panels, merged images for co-localization. E, immunofluorescence staining for ezrin and EGFR (top row) and ezrin and HER3 (bottom row) in SKBR3 cells at baseline in serum-containing medium. Right panels, merged images for co-localization. F, immunofluorescence staining of HER2 and Ezrin in SKBR3 cells treated with EGF or NRG1 for 2 h. Right panels, merged images for co-localization. G, co-immunoprecipitation for HER2 and ezrin in SKBR3 cells. H, quantitation of PLA assays using antibodies for HER2 and NHERF1 or HER2 and Ezrin in control SKBR3 cells or in SKBR3 cells treated with EGF for 10 min. n = 8 for HER2 and NHERF1 in control cells, n = 7 for all other conditions. J, Western blot analysis of Ezrin, HER2, and phospho-HER2 in control and EzrinKD SKBR3 cells. Bar graphs represent quantitation of three separate experiments. K, Western blot analysis of HER2 and phospho-HER2 in control and NRG668394-treated SKBR3 and BT474 cells. Bar graphs represent quantitation of three separate experiments. L, immunofluorescence staining of EzrinKD SKBR3 (left panel) and control SKBR3 cells treated with NRG668394 (right panel). Cells were stained for HER2 and phalloidin (top row), HER2 and Ezrin (center row), or HER2 and NHERF1 (bottom row). Enlarged Z stacks at the far right show internalization of HER2 within the cells (white arrows). M, the bar graphs represents percentages of cells with internalized HER2 in control (219 cells assessed), EzrinKD (68 cells assessed), and NRG668394-treated (104 cells assessed) SKBR3 cells. N, immunofluorescence staining for HER2 and EGFR (top row) or HER2 and HER3 (bottom row) in EzrinKD SKBR3 cells. Right panel, magnification of the boxed area in the *third panel* in each row. White arrows indicate co-localization of internalized HER2 with EGFR (top row) or HER3 (bottom row). Error bars represent mean ± S.E. for three experiments unless otherwise indicated. ***, p < 0.0005; ****, p < 0.00005. Scale bars = 10 μm.

**Ezrin stabilizes membrane HER2**

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Inhibition of ezrin activity causes internalization of HER2 in a PKCα-dependent fashion

In light of the similarities between inhibiting ezrin and activating PKCα, we next asked whether the ezrin inhibitor NSC668394 promotes HER2 internalization in a PKCα-dependent fashion. First, we transfected SKBR3 cells with HA-tagged WT-PKCα and then treated them with NSC668394. As shown in Fig. 5A, this led to internalization and co-localization of HER2 and WT-PKCα, mimicking the results seen with transfection of CA-PKCα as well as with treatment with PMA or calcium (Fig. 4). Next, we examined whether treatment with the PKCα inhibitor Go6974 affected the ability of NSC668394 to inhibit HER2 signaling or to promote HER2 internalization. As shown in Fig. 5B, treatment with Go6974 largely prevented the decrease in total HER2 and pHER2 levels caused by inhibition of ezrin activity. As noted previously, NSC668394 caused a reduction in membrane protrusions and promoted HER2 internalization. However, in the presence of Go6974, membrane protrusions were partially preserved, and HER2 internalization was inhibited (Fig. 5, C–E). Almost 50% of cells treated with NSC668394 had intracellular HER2 staining, but Go6974 decreased the percentage of cells with internalized HER2 almost to baseline (Fig. 5E). As shown in Fig. 5F, NSC668394 also diminished the ability to co-immunoprecipitate ezrin with HER2. Interestingly, even though Go6974 substantially blocked internalization of HER2, it did not restore the ability to co-immunoprecipitate HER2 and ezrin. Therefore, although inhibition of ezrin may disrupt interactions between ezrin and HER2 in a PKCα-independent fashion, activation of PKCα is required for efficient internalization of HER2 when it is separated from the signaling complex.

Ezrin stabilizes membrane HER2

HSP90 is required to maintain cell surface expression of HER2 and to stabilize membrane protrusions (7, 10–12). Therefore, we examined whether Ezrin and HSP90 interact to
Ezrin stabilizes membrane HER2

Regulate the cell surface localization of HER2. As reported previously, immunofluorescence staining of SKBR3 cells demonstrated co-localization of HER2 and HSP90 in membrane protrusions (Fig. 6A) (10, 11). Knocking down ezrin expression or inhibiting ezrin activity with NSC668394 decreased the intensity of HSP90 staining associated with the plasma membrane, disrupted the co-localization of HSP90 and HER2, and caused internalization of HER2 (Fig. 6A). Inhibiting ezrin also reduced the ability to co-immunoprecipitate HER2 with HSP90 (Fig. 6B). In a reciprocal fashion, inhibiting HSP90 also disrupted interactions between ezrin and HER2. Knocking down HSP90 in SKBR3 cells caused internalization of ezrin and NHERF1, which no longer co-localized with the internalized HER2 (Fig. 6C). However, immunofluorescence staining for ezrin and NHERF1 remained co-localized within the cells (Fig. 6C). Knocking down HSP90 was associated with a reduction in ezrin

Figure 5. A, immunofluorescence staining for HER2 and HA-tagged WT-PKCα in SKBR3 cells treated with NSC668394. The top right panels and bottom row represent a magnification of the boxed area in the top left panel. Insets on top and on the right represent Z stacks in two different orientations. White arrows indicate co-localization of internalized HER2 and PKCα. B, immunoblot analysis of HER2 and phospho-HER2 levels in lysates from SKBR3 cells at baseline and after treatment with PMA alone or a combination of PMA plus Go6974. C, percentage of SKBR3 cells forming membrane protrusions at baseline or after treatment with NSC668394 alone or a combination of NSC668394 and Go6974. D, immunofluorescence staining for HER2 in control SKBR3 cells (left column), SKBR3 cells treated with NSC668394 (center column), and SKBR3 cells treated with a combination of NSC668394 and Go6974 (right column). Bottom row, magnification of the boxed areas in the top row. Insets on top and on the right represent Z stacks in two different orientations. White arrows indicate HER2 at cell surface protrusions. The yellow arrow indicates internalized HER2. E, percentage of cells with internalized HER2. F, co-immunoprecipitation of HER2 and Ezrin from control SKBR3 cells and from SKBR3 cells treated with NSC668394 alone or a combination of NSC668394 and Go6974. The bar graph represents the relative levels ezrin in IP for HER2 (corrected for total HER2). Error bars represent mean ± S.E. for three experiments. ****, p < 0.0005. Scale bars = 10 μm.

Figure 4. A, co-immunoprecipitation of HER2 and Ezrin from control and PMA-treated or bryostatin 1–treated SKBR3 cells. Bar graphs indicate the relative levels of ezrin in IP for HER2 (corrected for total HER2). B, immunoblot analysis of HER2 and phospho-HER2 levels in SKBR3 cells at baseline or after treatment with PMA or bryostatin 1. Bar graphs indicate the relative levels of phospho-erzrin. C, immunoblot analysis of Ezrin and phospho-Ezrin levels in SKBR3 cells at baseline or after treatment with PMA or bryostatin 1. Bar graphs indicate the relative levels of phospho-erzrin. D, immunofluorescence staining for HER2 and Ezrin in SKBR3 cells incubated with PMA (top row) and bryostatin 1 (bottom row). The three right panels represent a magnification of the boxed area in the left panel. Insets on top and on the right represent Z stacks in two different orientations. Far right panels, magnified Z stacks. White arrows indicate internalized Ezrin, and yellow arrows represent internalized HER2. E, immunofluorescence staining for HER2 and NHERF1 in SKBR3 cells incubated with PMA (top row) and bryostatin 1 (bottom row). The three right panels represent a magnification of the boxed area in the left panel. Far right panels, magnified Z stacks. White arrows indicate internalized Ezrin, and yellow arrows represent internalized HER2. F, immunofluorescence staining of NHERF1 and Ezrin in SKBR3 cells incubated with PMA (top row) and bryostatin 1 (bottom row). The three right panels represent a magnification of the boxed area in the left panel. Far right panels, magnified Z stacks. White arrows indicate internalized Ezrin, and yellow arrows represent internalized HER2. G, percentage of cells forming membrane protrusions in control SKBR3 cells and in SKBR3 cells expressing either WT-PKCα or CA-PKCα. J, immunofluorescence staining of HER2 (top row), ezrin (center row), and NHERF1 (bottom row) in SKBR3 cells expressing HA-tagged WT-PKCα. The three right panels represent a magnification of the boxed area in the left panel. Far right panels, magnified Z stacks. White arrows indicate co-localization of HER2, NHERF1, or Ezrin with CA-PKCα. L, immunofluorescence staining of HER2 in SKBR3 cells expressing WT-PKCα at baseline (top row), and after treatment with PMA (center row) or 10 mM calcium and ionomycin (bottom row). The three right panels represent a magnification of the boxed area in the left panel. Far right panels, magnified Z stacks. White arrows indicate co-localization of internalized HER2, NHERF1, or Ezrin with CA-PKCα. M, immunofluorescence staining of HER2 in SKBR3 cells expressing WT-PKCα at baseline (top row) and after treatment with PMA (center row) or 10 mM calcium and ionomycin (bottom row). The three right panels represent a magnification of the boxed area in the left panel. Far right panels, magnified Z stacks. White arrows indicate co-localization of internalized Ezrin and PKCα in membrane protrusions (top row) or internalized into the cell (center and bottom rows). M, immunofluorescence staining of HER2 in SKBR3 cells expressing WT-PKCα at baseline (top row) and after treatment with PMA (center row) or 10 mM calcium and ionomycin (bottom row). The three right panels represent a magnification of the boxed area in the left panel. Far right panels, magnified Z stacks. White arrows indicate co-localization of internalized Ezrin and PKCα in membrane protrusions (top row) or internalized into the cell (center and bottom rows). Error bars represent mean ± S.E. for three experiments. **, p < 0.005; ***, p < 0.0005; ****, p < 0.00005. Scale bars = 10 μm.
phosphorylation as well as a decrease in the ability to co-immunoprecipitate ezrin with HER2 (Fig. 6, D and E). Similar results were seen when control SKBR3 cells were treated with the HSP90 inhibitor geldanamycin for 16 h (Fig. 6, F–H). Geldanamycin led to loss of membrane protrusions and internalization of HER2, ezrin, and NHERF1, with continued co-localization between NHERF1 and ezrin but loss of co-localization of HER2 with either ezrin or NHERF1 (Fig. 6, F–H). It also reduced the relative ability to co-immunoprecipitate ezrin with HER2 (Fig. 6I). These data demonstrate that HSP90 participates in interactions between ezrin, NHERF1, and HER2 that stabilize HER2 at the cell surface.
Inhibition of Ezrin affects cell proliferation and death and synergizes with lapatinib in a PKCα-dependent manner

Because inhibiting ezrin activity led to internalization of HER2 and reduced pHER2 levels, we reasoned that the ezrin inhibitor NSC668394 might also inhibit cell proliferation and/or enhance cell death in HER2-positive breast cancer cells. As predicted, treatment of SKBR3 cells or BT474 cells with NSC668394 inhibited proliferation (BrdU incorporation) and increased apoptosis in both cell lines (Fig. 7, A and B). Given our observations that the effects of NSC668394 on HER2 appeared to be mediated, in part, by activation of PKCα, we also treated SKBR3 and BT474 cells with PMA, which inhibited proliferation and increased apoptosis (Fig. 7, C and D). Consistent with an effect of PKCα, simultaneous treatment with Go6976 blunted the effects of both NSC668394 and PMA on BrdU incorporation and apoptosis in both cell lines (Fig. 7, A–D).

Lapatinib is a dual kinase inhibitor that targets HER2 and EGFR and is used to treat patients with HER2-positive breast cancer (47, 48). We examined whether inhibiting ezrin activity might be able to augment the effects of lapatinib treatment to inhibit cell proliferation and/or promote cell death in these two cell lines. As shown in Fig. 7, E and F, the combination of lapatinib and NSC668394 was more effective at inhibiting proliferation and promoting cell death than lapatinib alone. Furthermore, Go6976 blunted the additive effects of NSC668394 on SKBR3 and BT474 cell proliferation and apoptosis but not the baseline effects of lapatinib on these parameters (Fig. 7, E and F), consistent with the idea that blocking ezrin function may modulate cell turnover through activation of PKCα.

Discussion

The internalization and recycling or degradation of receptor tyrosine kinases are highly regulated processes that shape downstream signaling events (6, 8). Unlike other ErbB family members, upon activation, ErbB2/HER2 resists internalization and degradation and tends either to be quickly recycled or to remain at the cell surface, where it can continue to signal for prolonged periods (6–9). Interactions between HER2 and HSP90 as well as the localization of HER2 within microvillus-like protrusions have been described previously to contribute to its plasma membrane stability in breast cancer cells (7, 10, 12, 49–51). Work from our laboratory has shown that assembly of a complex including HER2, its signaling partners EGFR or HER3, the calcium ATPase PMCA2, the scaffolding molecule NHERF1, and the chaperone HSP90 within actin-rich and lipid raft–rich membrane protrusions contributes to retention of activated HER2 on the cell surface and stimulation of Akt signaling (10, 11, 52). In this report, we extend prior findings from our laboratory and others (31, 32) by demonstrating that ezrin contributes to stabilizing this multiprotein signaling complex within specific membrane domains and, in doing so, prevents HER2 internalization. We find that ezrin expression is up-regulated and co-localizes with HER2 in hyperplastic lesions and tumors from MMTV-Neu mice as well as in human DCIS. Ezrin mRNA levels correlate with HER2 and NHERF1 mRNA levels in invasive breast cancers from patients, and, in HER2-positive breast cancer cell lines, ezrin co-immunoprecipitates and/or co-localizes with HER2 and NHERF1. Genetic or pharmacologic disruption of ezrin function leads to internalization of HER2 and loss of co-localization between HER2 and NHERF1.
These findings suggest the working hypothesis outlined in Fig. 8. We propose that the HER2/EGFR and HER2/HER3 heterodimers interact with PMCA2 and HSP90 at the cell membrane and, in turn, that PMCA2 interacts with NHERF1 through its C-terminal PDZ recognition sequence. We hypothesize that ezrin then connects NHERF1, PMCA2, HSP90, and HER2 to the actin cytoskeleton within membrane protrusions that are enriched for lipid rafts and PIP2, stabilizing the complex within this specialized membrane domain and preventing HER2 from moving away from these structures to become ubiquitylated and internalized (10, 11). Inhibition of HER2 tyrosine kinase activity or genetic or pharmacologic targeting of PMCA2, NHERF1, or ezrin disrupts interactions between PMCA2, HER2, NHERF1, and ezrin, interfering with interactions between HER2 and HSP90, promotes remodeling of the plasma membrane structure and triggers PKCα-mediated internalization of HER2, eventually leading to degradation of HER2. When combined, these series of observations and our previous work provide a high degree of confidence that activation of HER2 signaling brings together multiprotein complexes that include HER2/EGFR or HER2/HER3 heterodimers as well as PMCA2, HER2, NHERF1, ezrin, HSP90, and likely other molecules, all within actin-, lipid raft- and PIP2-enriched portions of the membrane. In these regions, the membrane structure, the constituent proteins, and active HER2 signaling all seem to reinforce each other and contribute to the retention of activated signaling complexes at the cell surface. Inhibiting HER2 kinase activity or knocking down or inhibitig any of the above constituent proteins always leads to the combination of alterations in plasma membrane structure, disruption of the multiprotein complex, loss of HER2 phosphorylation, and internalization of HER2 itself (10, 11, 32, 52). Future studies will be required to fully understand how biochemical signaling, membrane lipids, and actin remodeling are integrated at sites of active HER2 signaling.

Increased expression and/or abnormal localization of ezrin have been associated with breast cancer progression, increased HER2 expression, and a poor prognosis in patients (15, 27, 29, 31, 33, 34). We found that normal mammary ducts, like other polarized epithelia, express only low levels of ezrin specifically at the apical membrane (27, 30). Furthermore, we found that up-regulation and basolateral extension of ezrin expression appear to be early abnormalities during the development of tumors in MMTV-Neu mice because it occurs in cells within precancerous hyperplastic lesions. We also observed basolateral extension of ezrin staining in HER2-positive human DCIS.

Figure 8. A, working model for a membrane- and actin-tethered multiprotein signaling complex containing ezrin, NHERF1, PMCA2, HSP90, and heterodimers of HER2/EGFR or HER2/HER3 in lipid raft-rich membrane protrusions. B, PKCα-mediated disruption of interactions between NHERF1 and PMCA2 causes disassembly of the signaling complex, effacement of membrane protrusions, and internalization of ezrin–NHERF1 and PMCA2–HER2–EGFR–HER3 complexes.
**Ezrin stabilizes membrane HER2**

Although this change in ezrin localization most likely reflects a breakdown of polarity in these cells, we did not observe significant ezrin staining in HER2-negative DCIS, only in lesions with elevated levels of HER2. In normal epithelial cells, HER2 is restricted to the basolateral surface because of its interaction with a scaffolding protein called erbin (53, 54). However, in hyperplasia, DCIS lesions, and invasive cancers, we found that HER2 was located throughout the plasma membrane, where it co-localized with ezrin. These results lead us to speculate that a breakdown of cell polarity may allow HER2 to mix with NHERF1, ezrin, and PMCA2, which are usually restricted to the apical surface of mammary epithelial cells. These novel interactions might then stabilize HER2 at the plasma membrane and increase HER2 protein expression and signaling, contributing to tumor progression. Consistent with this idea, Sarrío et al. (34) examined a series of 509 breast cancers and found that mislocalization of ezrin staining away from a purely apical distribution correlated with more aggressive tumor characteristics and that uniform membranous staining for ezrin was associated with elevated HER2 expression and high levels of phospho-AKT staining (34). Likewise, in a series of 75 patients, Antelmi et al. (31) found that activated phospho-ezrin always localizes to the plasma membrane and that its staining intensity correlates with higher tumor grade, HER2 expression, and a worse prognosis.

We have shown that knocking down the expression of PMCA2, NHERF1, HSP90, or HER2 is associated, in each case, with an increase in intracellular calcium levels, presumably secondary to reduced expression of PMCA2 (10, 11, 52). We now propose that the effects of elevated intracellular calcium are mediated, at least in part, by activation of protein kinase Ca. Our experiments suggest that PKCa co-localizes with HER2, ezrin, and NHERF1 within specific membrane domains and that, when activated either pharmacologically, genetically, or with intracellular calcium, it triggers dissociation of ezrin and NHERF1 from HER2 and promotes HER2 internalization. These data are in agreement with prior studies demonstrating that HER2 and PKCa co-localize at the plasma membrane and in intracellular endosomes and that activation of PKCa can stimulate internalization of HER2 and inhibit HER2 signaling (41, 45, 55, 56). A task for future investigation will be to define the molecular targets of PKCa involved in disrupting ezrin/NHERF1–HER2 interactions and in promoting their subsequent internalization.

In conclusion, the results in this report suggest an important role for ezrin in maintaining a multiprotein complex required for high levels of actively signaling HER2 on the surface of breast cancer cells. The different members of this signaling complex, which include ezrin, NHERF1, PMCA2, HSP90, EGFR or HER3, and HER2, appear to be involved in a series of interdependent interactions that stabilize and amplify HER2 expression and signaling within specific plasma membrane domains. Experience with HSP90 inhibitors has already suggested that disrupting the membrane stabilization of HER2 has clinical utility (12, 57). Therefore, targeting other members of this complex singly or in combination might also be efficacious for the treatment of HER2-positive cancers. Although preliminary, our experiments combining an ezrin inhibitor with lapatinib support this idea. By better understanding the molecular details through which members of this complex interact, it may be possible to devise novel ways to target HER2 signaling in a cancer cell–specific fashion.

**Experimental procedures**

**Materials**

We obtained the Ezrin inhibitor NSC668394 from Calbiochem, the HSP90 inhibitor geldanamycin (G3381) from Sigma-Aldrich, the PKC agonist PMA (sc-3576) and the dual EGFR/HER2 kinase inhibitor lapatinib (sc-353668) from Santa Cruz Biotechnology, the PKC agonist bryostatin 1 (2383) from Tocris, and the PKCa inhibitor Go6976 (ab141413) from Abcam.

**Cell culture**

The human cell lines SKBR3 and BT474 were obtained from the ATCC and maintained in culture in Dulbecco’s modified Eagle’s medium and GlutaMAX-1 (Gibco Life Technologies) containing 10% fetal bovine serum and penicillin/streptomycin (Gibco Life Technologies) at 37 °C in 5% CO2. MCF10A cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (Gibco Life Technologies) containing 5% horse serum, EGF (100 μg/ml), hydrocortisone (1 mg/ml), cholera toxin (1 mg/ml), insulin (10 mg/ml), and penicillin/streptomycin (Gibco Life Technologies) at 37 °C in 5% CO2. In some experiments, cells were cultured as above but in medium without fetal bovine serum for 16 h and then treated with 100 ng/ml EGF (Cell Signaling Technology) or 50 ng/ml NRG1 (Cell Signaling Technology) for 2 h. In other experiments, various pharmacologic agents were added to medium for 16 h before harvesting cells; these included NSC668394 at 5 μM, PMA at 100 nM, bryostatin 1 at 250 nM, Go6976 at 2 μM, geldanamycin at 2 μM, and lapatinib at 2 μM. To raise intracellular calcium levels, cells were exposed to 10 mM extracellular calcium and 1 μM ionomycin for 16 h.

**RNA extraction and real-time RT-PCR**

RNA was isolated using TRIzol (Invitrogen). Quantitative RT-PCR was performed with the SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen) using a Step One Plus Real-Time PCR System (Applied Biosystems) and the following TaqMan primer sets: Ezrin and Hs00931646_m1. Human HPRT1 (4326321E, Invitrogen) was used as a reference gene. Relative mRNA expression was determined using Step One software v2.2.2 (Applied Biosystems).

**Cell transfections**

Constructs encoding GFP-C1-PLCδ-PH (plasmid 21179), HA-tagged WT-PKCa (plasmid 21232), and HA-tagged constitutively active PKCa (plasmid 21233) are commercially available from Addgene (Cambridge, MA). SKBR3 cells were transfected using FuGENE 6 transfection reagent (Invitrogen) according to the manufacturer’s instructions.

**Knockdown cell lines**

A stable cell line expressing shRNA directed against Ezrin was generated by transducing cells with commercially prepared
lentiviruses containing three individual shRNAs directed against ezrin mRNA: Ezrin (sc-35349-V, Santa Cruz Biotechnology). Briefly, cells were cultured in 12-well plates and infected by adding the shRNA lentiviral particles to the culture for 48 h according to the manufacturer’s instructions. Stable clones expressing the specific shRNAs were selected using 5 μg/ml of puromycin (Gibco Life Technologies) and pooled to generate the cells used in the experiments. Knockdown cells for PMCA2 and NHERF1 were described previously (10, 11).

**Immunofluorescence**

Cells were grown on coverslips, fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 10 min, washed three times with PBS, and incubated with primary antibody overnight at 4 °C. The cells were then washed three times with PBS and incubated with secondary antibody for 1 h at room temperature. After washing, coverslips were mounted using Prolong Gold antifade reagent with DAPI (Invitrogen). Paraffin-embedded tissue sections were cleared with histoclear (National Diagnostics) and graded alcohol using standard techniques. Antigen retrieval was performed using 7 mM citrate buffer (pH 6.0) under pressure. Sections were incubated with primary antibody overnight at 4 °C and with secondary antibody for 1 h at room temperature. Coverslips were mounted using Prolong Gold antifade reagent with DAPI (Invitrogen). All images were obtained using a Zeiss 780 confocal microscope. Primary antibodies included those against HER2 (sc-284), NHERF1 (sc-134485), and the HA probe (sc-7392) from Santa Cruz Biotechnology (Dallas, Texas); HER2 (MA1-35720) from Thermo Scientific (Waltham, MA); Ezrin (31455S), phospho-Ezrin (Thr-567, 3141S), phospho-HER2 (Thr-1221/1222, 2243S), EGFR (4267S), HER3 (12708P), and the HA probe (sc-7392) from Invitrogen. We also stained for actin using phalloidin-Atto 488 (49409) from Sigma. Sections from normal mouse mammary glands and tumors from MMTV-Neu transgenic mice were obtained following protocols reviewed and approved by the Yale Institutional Animal Care and Use Committee.

The proportion of cells with membrane protrusions or with internalized HER2 was quantified on Z stack images of HER2-stained cells using a Zeiss 780 confocal microscope. Membrane protrusions were identified by HER2 staining forming a punctate extension above the outline of the apical cell surface on Z stack images. Internalization of HER2 was scored as HER2 staining within the cytoplasm of the cell, as defined by a location between the apical surface of the cell and the basal surface of the cell as defined by Z stack reconstructions. In each case, at least 68 cells were randomly examined, and the percentage of all counted cells demonstrating evidence of protrusions or internalized HER2 was tallied.

**Co-immunoprecipitation**

Cells were incubated in PBS containing 20 mM of the cross-linking reagent succinimidyl 6-((2-(pyridyldithio))propionamido)hexanoate (catalog no. 21651, Thermo Scientific) for 1 h at room temperature and then lysed with radioimmune precipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM Tris-HCl, and 150 mM NaCl). Cell extracts were subsequently incubated overnight at 4 °C with protein A/G beads (sc-2003, Santa Cruz Biotechnology) and the specific antibody. After centrifugation, the immunoprecipitated proteins were eluted with lithium dodecyl sulfate sample buffer containing 10% β-mercaptoethanol to reverse the cross-linking. The resulting samples were then analyzed by Western blotting (10). Co-IP experiments were quantified by determining the density of specific bands using the Odyssey IR imaging system (LI-COR) densitometer and correcting the densitometric intensity of the co-immunoprecipitated protein for any variation in the densitometric intensity of the bands corresponding to the initially immunoprecipitated protein between experimental conditions. Each experiment was repeated at least three times, and results were expressed as the amount of co-immunoprecipitated ezrin or HER2 in experimental cells relative to baseline in control cells.

**Immunoblotting**

Protein samples were prepared from cells using standard methods, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane by wet Western blot transfer (Bio-Rad). The membrane was blocked in TBST buffer (Tris-buffered saline and 1% Tween) containing 5% milk for 1 h at room temperature. The blocked membranes were incubated overnight at 4 °C with specific primary antibodies (Odyssey blocking buffer, 927-40000). The membranes were washed three times with TBST buffer and then incubated with specific secondary antibodies provided by LI-COR for 2 h at room temperature. After three washes with TBST buffer, the membranes were analyzed using the Odyssey IR imaging system (LI-COR). All immunoblot experiments were performed at least three times, and representative blots are shown in the figures. Quantitation represents the mean densitometric intensity of the specific bands corrected for any variation in β-actin intensity with the experimental condition expressed relative to baseline in control conditions/cells.

**Proximity ligation assays**

PLAs (39) were performed using the Duolink™ assay kit (Sigma). SkBR3 cells were seeded onto 22-mm round collagen-coated coverslips (Corning, catalog no. 354089). The experiments were performed when the cells reached 80% confluence. Cells were serum-starved the night before the experiments and stimulated with EGF (100 ng/ml final concentration) for 10 min the next morning. The cells were washed three times with PBS, and paraformaldehyde (4% in PBS) was added to each well for 15 min; then they were permeabilized with 0.1% Triton X-100 in PBS. Permeabilized cells were incubated with combinations of the following antibodies: rabbit anti-HER2 (Santa Cruz Biotechnology, sc-284), rabbit anti-NHERF1 (Abcam, ab9526), and mouse monoclonal anti-Ezrin (Invitrogen, N MA5-13862). PLA probes were then added, and the assay was performed according to the manufacturer’s instructions. Images were acquired in 12 z-planes, and PLA signals were normalized to the number of nuclei using Imaris software in the Department of Cell Biology, University of Pittsburgh. Results are expressed as the absolute number of PLA dots per nucleus (cell).
Ezrin stabilizes membrane HER2

RNA extraction and real-time RT-PCR

RNA was isolated using TRIzol (Invitrogen). Quantitative RT-PCR was performed with the SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen) using a Step One Plus Real-Time PCR System (Applied Biosystems), and the TaqMan primer set (Ezrin, Hs00931658_g1) was used as reference genes (Invitrogen). Relative mRNA expression was determined using Step One software v2.2.2 (Applied Biosystems).

Cell proliferation and apoptosis

Cell proliferation was assessed by measuring BrdU incorporation using the Cell Proliferation ELISA Kit (11647229001) from Roche. Apoptosis was measured using the Cell Death Detection ELISA Kit (11544675001) from Roche (Genentech Inc.).

Statistics

Statistical analyses were performed with Prism 6.0 (GraphPad Software, La Jolla, CA). Statistical significance was determined using unpaired t test for comparisons between two groups and one-way analysis of variance for groups of three or more. Mutual exclusion analysis on the METABRIC data was performed using tools within the CBioPortal for Cancer Genomics website (http://www.cbioportal.org)3 (35, 37). Correlations between ezrin, NHERF1, and HER2 were determined by downloading primary gene expression array data from the METABRIC data using the cBioPortal website (35, 37). These data were entered into Prism 6.0 software, and XY correlations were performed using Prism software.

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