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Calmodulin binds HER2 and modulates HER2 signaling ☆

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Human epidermal growth factor receptor 2 (HER2), a member of the ErbB family of receptor tyrosine kinases, has defined roles in neoplastic transformation and tumor progression. Overexpression of HER2 is an adverse prognostic factor in several human neoplasms and, particularly in breast cancer, correlates strongly with a decrease in overall patient survival. HER2 stimulates breast tumorigenesis by forming protein–protein interactions with a diverse array of intracellular signaling molecules, and evidence suggests that manipulation of these associations holds therapeutic potential. To modulate specific HER2 interactions, the region(s) of HER2 to which each target binds must be accurately identified. Calmodulin (CaM), a ubiquitously expressed Ca^{2+} binding protein, interacts with multiple intracellular targets. Interestingly, CaM binds the juxtamembrane region of the epidermal growth factor receptor, a HER2 homolog. Here, we show that CaM interacts, in a Ca^{2+}-regulated manner, with two distinct sites on the N-terminal portion of the HER2 intracellular domain. Deletion of residues 676–689 and 714–732 from HER2 prevented CaM–HER2 binding. Inhibition of CaM function or deletion of the CaM binding sites from HER2 significantly decreased both HER2 phosphorylation and HER2-stimulated cell growth. Collectively, these data suggest that inhibition of CaM–HER2 interaction may represent a rational therapeutic strategy for the treatment of patients with breast cancer. This article is part of a Special Issue entitled: 11th European Symposium on Calcium.

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1. Introduction

The ErbB family of transmembrane receptors has four members, namely epidermal growth factor receptor (EGFR)/HER1/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4 [1]. Each member is a typical receptor tyrosine kinase, comprising an extracellular ligand binding region, a single membrane-spanning region, and a cytoplasmic C-terminal region that contains the tyrosine kinase domain. ErbB receptors control intracellular signaling pathways that govern fundamental cellular processes, including proliferation, migration, metabolism and survival. They therefore have important roles during development and in normal adult physiology [2]. In addition, EGFR and HER2 have been implicated in the pathogenesis of several types of human cancer [3]. For example, HER2 is overexpressed in 20–25% of invasive breast cancers [4], and increased HER2 expression correlates strongly with a shorter time to relapse and a decrease in overall survival [5].

Unlike other members of the ErbB family, HER2 does not bind a specific ligand directly. Instead, it exists in a constitutively phosphorylated state [6,7]. Active HER2 forms protein–protein complexes with a diverse array of intracellular signaling molecules and, through these interactions, stimulates the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) cascades [8]. Activation of these pathways downstream of HER2 results in increased cell proliferation, transformation and oncogenesis [8]. Recently, increasing effort has been directed towards identifying methods to overcome HER2-stimulated carcinogenic signaling. Evidence suggests that the intracellular proteins to which HER2 binds are, at least in part, responsible for its transforming ability [8,9]. Manipulation of the interactions between HER2 and these targets therefore holds potential for anti-cancer therapy [9]. For specific manipulation to become possible, the region(s) on HER2 to which each protein binds must be accurately identified.

Calmodulin (CaM) is a small Ca^{2+} binding protein that interacts with multiple intracellular targets. Although the best characterized of these are the serine/threonine kinases [10], a large body of literature has revealed several "non-classic" CaM binding partners [11]. The discovery of these proteins, which include cell surface receptors such as the insulin receptor [12,13], steroid receptors such as estrogen receptor α (ERα) [14–16], and ion channels such as the Ca^{2+}-gated K^+ channel [17], has substantially increased the number of physiological and pathological processes known to be regulated by CaM. Of particular interest is the fundamental role CaM now appears to play in breast

Abbreviations: CaM, calmodulin; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; ERα, estrogen receptor α; GST, glutathione S-transferase; HER2, human epidermal growth factor receptor 2; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PVDF, polyvinylidene fluoride

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carcinogenesis. For example, CaM binds directly to ERα [14–16] (which stimulates tumorigenesis in ER(+) breast carcinoma [18]), and tamoxifen, a triphenylethylenic antiestrogen used for the treatment of steroid receptor-positive breast cancer [19], modulates CaM–ERα binding [14,16,20].

CaM is known to interact with ErbB family members. Initial analyses suggested that the cytosolic juxtamembrane region of EGFR, specifically residues 645–660, regulated Ca^{2+}-dependent CaM binding [21]. Later reports confirmed these findings [22,23], and deletion of this region was shown to result in a mutant receptor with no detectable EGF-stimulated kinase activity despite an intact ligand binding capacity [24–26]. One study suggested that CaM also binds HER2 [27], but this observation has not been independently validated. Moreover, the region of HER2 with which CaM interacts and the functional consequences of CaM binding on HER2-stimulated tumorigenesis remain undefined. Here, we identify the CaM binding sites on HER2, and show that antagonizing CaM function or disrupting CaM–HER2 association inhibits both HER2 phosphorylation and HER2-stimulated cell growth. Collectively, these data suggest that inhibition of CaM–HER2 interaction may be a potential approach for treating patients with breast cancer.

2. Materials and methods

2.1. Materials

SkBR3 and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA). All tissue culture reagents were obtained from Gibco (Carlsbad, CA). Anti-phospho-HER2 (Tyr^{1221}/Tyr^{1222}), anti-HER2 and anti-β-Tubulin antibodies, and a glutathione S-transferase (GST) fusion construct encoding the entire HER2 intracellular domain (designated GST-HER2; residues 676–1255 [numbering corresponds to the immature sequence]) were obtained from Cell Signaling Technology (Danvers, MA). CaM–Sepharose and secondary antibodies for enhanced chemiluminescence (ECL) detection were obtained from GE Healthcare (Piscataway, NJ). Pure CaM was obtained from Ocean Biologics (Edmonds, WA). The highly specific anti-CaM monoclonal antibody has been previously characterized [28]. CGS9343B was obtained from Tocris Biosciences (Ellisville, MO). Unless otherwise stated, all other reagents were of standard analytical grade.

2.2. Preparation of constructs and fusion proteins

GST-HER2 was expressed in *Escherichia coli* and isolated using glutathione-Sepharose essentially as previously described [29]. To construct GST-HER-N, GST-HER2-M, GST-HER2-C and GST-HER2Δ1, PCR was performed using GST-HER2 as a template with the following primers: GST-HER2-N: 5′-GAAGATCTAAGCGACGGCAGCAGAAGATCC-3′ (F), 5′-GCTCTAGACTCGAGTCACCGACATTCAGAGTCAATC-3′ (R); GST-HER2-M: 5′-GAAGATCTGACCTGCTGAACTGGTGTATGC-3′ (F), 5′-GCTCTAGACTCGAGTCACTGTAGAGGGCTGGGGTCATG-3′ (R); GST-HER2-C: 5′-GAAGATCTCCAAGATTCCGGGAGTTGGTG-3′ (F), 5′-GCTCTAGACTCGAGTCACACTGGCACGTCCAGACCC-3′ (R); GST-HER2Δ1: 5′-GAAGATCTCTGCTGCAGGAAACGGAGC-3′ (F), 5′-GCTCTAGACTCGAGTCACCGACATTCAGAGTCAATC-3′ (R). All forward primers included the BGLII restriction site and all reverse primers included the XhoI restriction site. The product was cut with BGLII and XhoI and subcloned into the BGLII–XhoI restriction sites of pGEX4T-1. To construct GST-HER2Δ2 and GST-HER2Δ3, PCR was performed using GST-HER2-N as a template with the following primers: GST-HER2Δ2: 5′-phos-ACAGTCTACAAGGGCATCTGG-3′ (F), 5′-phos-CCGCATCTGCGCCTGGTTGGG-3′ (R); GST-HER2Δ3: 5′-phos-AGTGATGTGTGGAGTTATGGTG-3′ (F), 5′-phos-CCCATCTGCATGGTACTCTGTC-3′ (R). GST-HER2Δ1,3 and GST-HER2Δ1,2,3 were constructed using sequential PCR reactions and appropriate template with the primers listed above. To construct GST-HER2-1, PCR was performed to anneal the following primers: 5′-GATCTAAGCGACGGCAGCAGAAGATCCGGAAGTACACGATGCGGAGATGAG-3′ (F), 5′-AATTCTCATCTCCGCATCGTGTACTTCCGGATCTTCTGCTGCCGTCGCTTA-3′ (R). GST-HER-1,2, GST-HER2-2 and GST-HER2-3 were constructed using PCR with pcDNA3-HER2 as a template and the following primers: GST-HER2-1,2: 5′-GAAGATCTAAGCGACGGCAGCAGAAGATCC-3′ (F), 5′-CGGAATTCTCAGCCAAAAGCGCCAGATCC-3′ (R); GST-HER2-2: 5′-GAAGATCTATCCTGAAAGAGACGGAG-3′ (F), 5′-CGGAATTCTCAGCCAAAAGCGCCAGATCC-3′ (R); GST-HER2-3: 5′-GAAGATCTGGCAAGGTGCCCATC-3′ (F), 5′-CGGAATTCTCACTGGTGGGTGAACCG-3′ (R). All forward primers included the BglII restriction site and all reverse primers included the EcorI restriction site. The product was cut with BglII and EcorI and subcloned into the BamHI–EcorI restriction sites of pGEX4T-1. To construct full-length HER2Δ1 and full-length HER2Δ2 (designated $HER2Δ1_{FL}$ and $HER2Δ2_{FL}$, respectively), pBLUESCRIPT-II-HER2Δ1 and pBLUESCRIPT-II-HER2Δ2, respectfully, were cut with EcorI and the product was subcloned into the EcorI restriction sites of pcDNA3-HER2. Prior to their inclusion in experiments, the sequences of all constructs were confirmed by dye-terminator sequencing.

2.3. In vitro binding assays

For *in vitro* binding experiments using pure proteins, pure CaM was incubated with pure GST-HER2 (residues 676–1255), GST-HER2-N (residues 676–966), GST-HER2-M (residues 820–1110), GST-HER2-C (residues 967–1255), GST-HER2Δ1 (residues 676–966 with residues 676–689 deleted), GST-HER2Δ2 (residues 676–966 with residues 714–732 deleted), GST-HER2Δ3 (residues 676–966 with residues 883–902 deleted), GST–HER2Δ1,3 (residues 676–966 with residues 676–689 and 883–902 deleted), GST–HER2Δ1,2,3 (residues 676–966 with residues 676–689, 714–732 and 883–902 deleted), GST–HER2-1 (residues 676–689), GST-HER2-1,2 (residues 676–732), GST–HER2-2 (residues 714–732), GST–HER2-3 (residues 883–902) (all >90% pure) or GST alone in 500 µl Ca^{2+} buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$ and 1% (v/v) Triton X-100) or EGTA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA and 1% (v/v) Triton X-100) for 3 h at 4 °C. GST-bound complexes were isolated using glutathione-Sepharose beads, washed 6 times in the same buffer used in the incubation, resolved by SDS-PAGE and processed by Western blotting.

2.4. Cell culture and transfection

SkBR3 cells were maintained in McCoy's 5A Medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. HEK293 cells were maintained in Dulbecco's Modified Eagles Medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. Cultures were regularly confirmed to be free of mycoplasma contamination. Transfections were performed using Trans-IT (Mirus, Madison, WI) according to the manufacturer's instructions.

2.5. CaM–Sepharose pulldown assays

SkBR3 cells were plated in 100 mm dishes at a density of 5×10^6 cells/dish and allowed to attach overnight. The following day, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in Ca^{2+} buffer or EGTA buffer supplemented with 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (together designated buffer C+ and E+, respectively). CaM–Sepharose pull down assays were performed essentially as previously described [30]. Briefly, clarified cell lysates were equalized for protein concentration using the modified Bradford Assay (Bio-Rad Laboratories, Hercules, CA), and equal amounts of protein were incubated with CaM–Sepharose or GST alone in 500 µl buffer C+ or E+ for 3 h at 4 °C.

CaM–Sepharose-bound complexes were isolated by centrifugation, washed 6 times in the same buffer used in the incubation, resolved by SDS-PAGE and processed by Western blotting. GST-bound complexes were isolated using glutathione-Sepharose beads and washed and processed in the same way.

2.6. Measurement of HER2 phosphorylation and signaling

SkBR3 cells were plated in 6 well plates at a density of 2×10^5 cells/well and allowed to attach overnight. The following day, cells were serum-starved in the presence of vehicle (DMSO) or 10 μM CGS9343B for 48 h. HEK293 cells were plated in 6 well plates at a density of 1×10^5 cells/well and allowed to attach overnight. The following day, cells were transfected with 2.5 μg/well vector (pcDNA3), HER2, HER2$\Delta1_{FL}$ or HER2$\Delta2_{FL}$. 24 h after transfection, cells were serum-starved for 48 h. For lysis, cell monolayers were placed on ice, washed twice with ice-cold PBS and lysed in 50 μl/well lysis buffer (50 mM Tris (pH 8.0), 100 mM NaF, 30 mM $Na_4P_2O_7$, 2 mM Na_2MoO_4 and 2 mM Na_3VO_4) supplemented with 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. Clarified cell lysates were equalized for protein concentration using the modified Bradford Assay, resolved by SDS-PAGE and processed by Western blotting. HER2 phosphorylation was measured by probing blots with phospho-specific HER2 antibody (dilution 1:1,000). In all experiments, blots were stripped by incubating with stripping buffer (62.5 mM Tris (pH 6.8), 2% (w/v) SDS and 0.7% (v/v) β-mercaptoethanol) for 30 min at 50 °C, then reprobed with an antibody against total HER2 (dilution 1:1,000). All blots were also probed with anti-β-Tubulin antibody (dilution 1:2,000) to verify protein loading. Densitometry was performed using ImageJ (Version 1.43).

2.7. Measurement of HER2-stimulated cell growth

HER2-stimulated cell growth was measured using sulforhodamine B staining essentially as previously described [31]. Briefly, SkBR3 and HEK293 cells were plated, transfected if appropriate, and serum-starved as described in the previous paragraph. Cell monolayers were then placed on ice and 1 ml ice-cold 25% (w/v) trichloroacetic acid was added directly to the culture medium. Cells were left for 1 h at 4 °C prior to being stained with 0.4% (w/v) sulforhodamine B (in 1% (v/v) acetic acid). Protein-bound dye was dissolved in 10 mM Tris (pH 10.5) and absorbencies were read at 510 nm.

2.8. Statistical analysis

All experiments were repeated independently at least 3 times. Statistical significance was set at $p<0.05$ and analyses were performed using the Student's t test. In all figures, * denotes statistical significance from control cells.

3. Results

3.1. CaM binds HER2 in a Ca^{2+}-regulated manner

To determine whether CaM interacts with HER2 in a normal cell milieu, we lysed SkBR3 cells, a malignant human breast epithelial cell line that overexpresses HER2, and incubated the lysate with CaM–Sepharose in the presence or absence of Ca^{2+}. In the presence of Ca^{2+}, binding of endogenous HER2 in cell lysates to CaM–Sepharose is readily detected (Fig. 1). Chelating Ca^{2+} with EGTA substantially reduces, but does not abrogate, CaM–Sepharose–HER2 association. The specificity of the interaction is validated by the absence of HER2 from samples that were incubated with GST alone (Fig. 1). The amount of HER2 in all cell lysates was equivalent. These data reveal

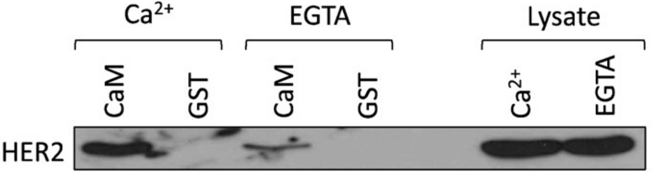


Fig. 1. CaM binds HER2 in a Ca^{2+}-regulated manner. CaM–Sepharose (CaM) or GST alone was incubated with equal amounts of protein from SkBR3 cell lysates in the presence (Ca^{2+}) or absence (EGTA) of Ca^{2+}. Complexes were isolated and washed as described in materials and methods. The samples were resolved by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane and probed with anti-HER2 antibody. An aliquot of each sample (equivalent to 2% of the amount in each pulldown) was also processed by Western blotting (Lysate). The data are representative of 5 independent experiments.

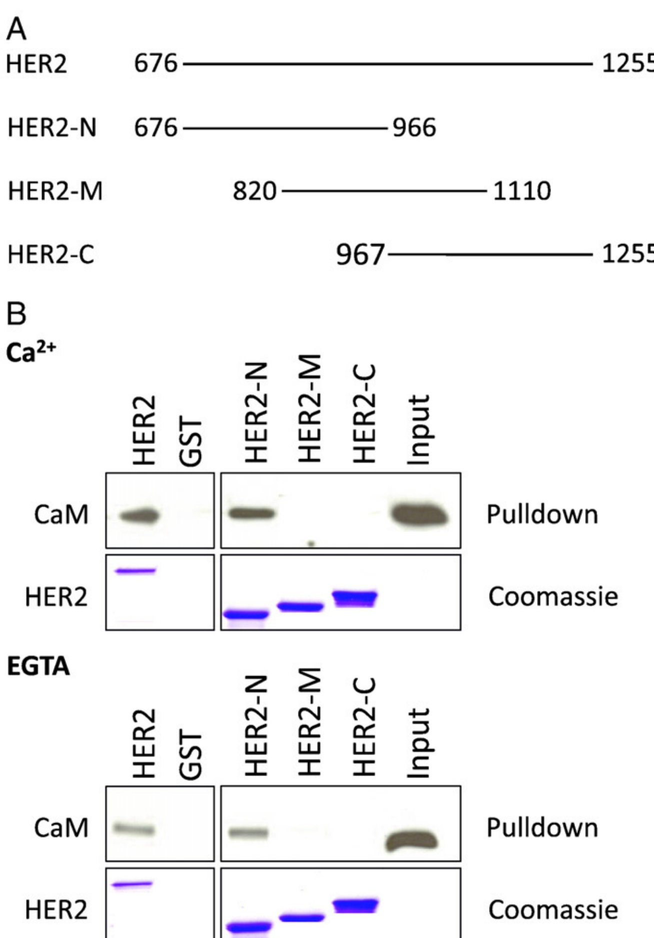


Fig. 2. Residues 676–966 of HER2 are necessary for CaM binding. A, Schematic representation of HER2 constructs depicting the HER2 intracellular domain and HER2 intracellular domain fragments. The specific residues present in each fragment are indicated. HER2, entire HER2 intracellular domain (residues 676–1255); HER2-N, N-terminal portion of HER2 intracellular domain (residues 676–966); HER2-M, middle fragment of HER2 intracellular domain (residues 820–1110); HER2-C, C-terminal portion of HER2 intracellular domain (residues 967–1255). B, Pure GST-HER2, GST-HER2-N, GST-HER2-M, GST-HER2-C or GST alone was incubated with equal amounts of pure CaM in the presence (Ca^{2+}) or absence (EGTA) of Ca^{2+}. Complexes were isolated and washed as described in the materials and methods and samples were resolved by SDS-PAGE. After running, the gel was cut at the 50 kDa marker. The bottom half was transferred to PVDF membrane and probed with anti-CaM antibody (Pulldown). The top half was stained with Coomassie to demonstrate essentially equal amounts of GST-HER2 in each sample (Coommassie). An aliquot of pure CaM (equivalent to 2% of the amount in each pulldown) was also resolved by SDS-PAGE and processed as described (Input). The data are representative of 5 independent experiments.

both a Ca^{2+}-dependent and a Ca^{2+}-independent interaction between CaM and HER2.

3.2. Identification of the CaM binding domain on HER2

The region of HER2 to which CaM binds was initially investigated using pure CaM and selected GST-tagged HER2 fragments (Fig. 2A). CaM was incubated with GST-HER2 constructs and GST-bound complexes were isolated, resolved by SDS-PAGE and processed by Western blotting. Ca^{2+}/CaM binds GST-HER2 but not GST alone (Fig. 2B). Examination of 3 peptides spanning the entire intracellular region of HER2 revealed that only the proximal N-terminal portion (HER2-N; residues 676–966) binds Ca^{2+}/CaM. No interaction between Ca^{2+}/CaM and the middle fragment (HER2-M; residues 820–1110) or the C-terminal portion (HER2-C; residues 967–1255) of HER2 was detected. The amount of apocalmodulin (Ca^{2+}-free CaM) that binds HER2 and HER2-N is less than that of Ca^{2+}/CaM (Fig. 2B). As expected, no apocalmodulin binds HER2-M or HER2-C. The amount of HER2 in each sample was essentially identical (Fig. 2B). Note that GST alone is not visible in the Coommassie images as the gel was cut at the 50 kDa marker and only the top half was stained. These data suggest that the region of HER2 containing residues 676–966 is necessary for binding both Ca^{2+}/CaM and apocalmodulin.

To narrow the CaM binding site, we analyzed the entire HER2 intracellular sequence using the Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html) and found 3 putative CaM interacting regions (Fig. 3). To investigate whether each of these sites is necessary for CaM–HER2 binding, we individually deleted each region from GST-HER2-N (residues 676–966; Fig. 3 and Fig. 4A). Deletion of either region 1 (HER2Δ1; residues 676–689 deleted) or 2 (HER2Δ2; residues 714–732 deleted) markedly attenuates the interaction of Ca^{2+}/CaM with HER2 (Fig. 4B). In contrast, deletion of the third region (HER2Δ3; residues 883–902 deleted) does not alter Ca^{2+}/CaM–HER2 binding. The last observation is not surprising as region 3 is present in both HER2-N and HER2-M (Fig. 3); HER2-M does not bind CaM (Fig. 2B). Deletion of regions 1 and 3 together (HER2Δ1,3; residues 676–689 and 883–902 deleted) considerably reduces, but does not abrogate, binding of HER2 to Ca^{2+}/CaM. In contrast, deletion of regions 1, 2 and 3 together (HER2Δ1,2,3; residues 676–689, 714–732 and 883–902 deleted) eliminates the ability of Ca^{2+}/CaM to associate with HER2.

In contrast to Ca^{2+}/CaM, apocalmodulin fails to bind HER2Δ1 (Fig. 4B). Interestingly, deletion of residues 714–732 (HER2Δ2) does not impair apocalmodulin binding. Binding of apocalmodulin to HER2Δ2, HER2-N and HER2Δ3 was essentially the same (Fig. 4B). Deletion of regions 1 and 3 together or 1, 2 and 3 together eliminates association of apocalmodulin with HER2. The specificity of the

680 690 700 710 720
KRRQQ KIRKYTMRRL LQETELVEPL TPSGAMPNQA QMR**ILKETEL**

730 740 750 760 770 780
RKVKVLGSGA FGTVYKGIWI PDGENVKIPV AIKVLRENTS PKANKEILDE AYVMAGVGSP

790 800 810 820 830 840
YVSRLLGICL TSTVQLVTQL MPYGCLLDHV RENRGRLGSQ DLLNWCMQIA KGMSYLEDVR

850 860 870 880 890 900
LVHRDLAARN VLVKSPNHVK ITDFGLARLL DIDETEYHAD **GGKVPIKWMA LESILRRRFT**

910 920 930 940 950 960
HQSDVWSYGV TVWELMTFGA KPYDGIPARE IPDLLEKGER LPQPPICTID VYMIMVKCWM

970 980 990 1000 1010 1020
IDSECRPRFR ELVSEFSRMA RDPQRFVVIQ NEDLGPASPL DSTFYRSLLE DDDMGDLVDA

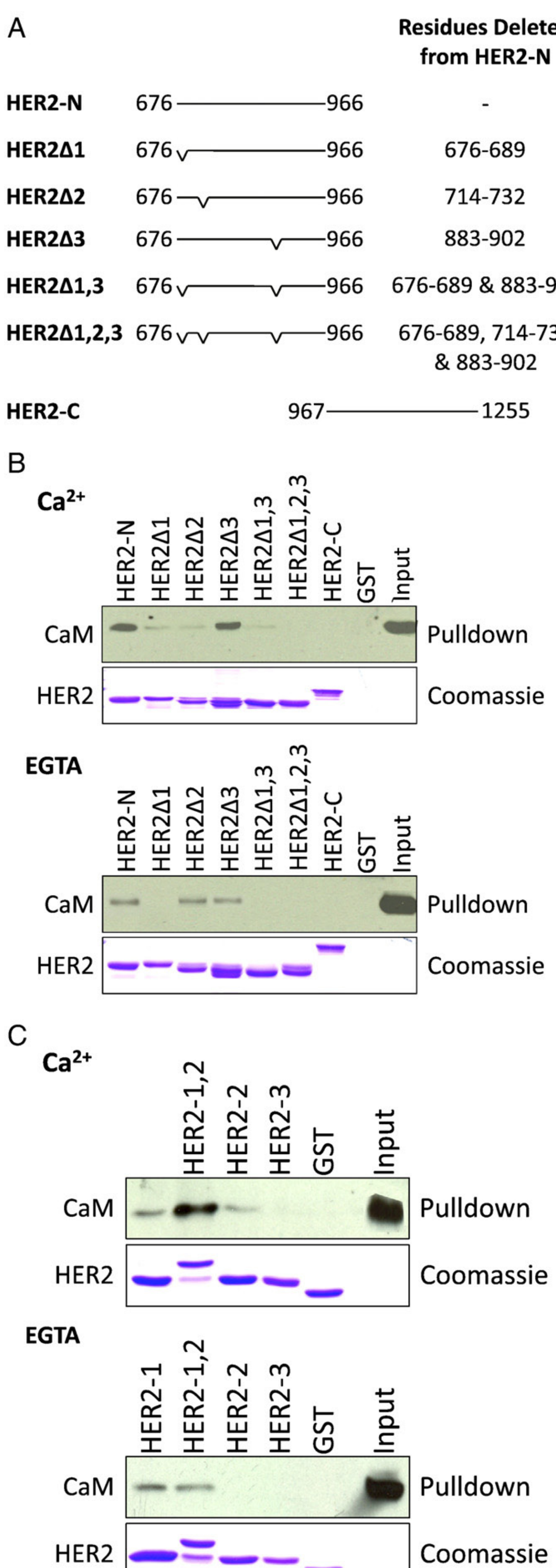
1030 1040 1050 1060 1070 1080
EEYLVPQQGF FCPDPAPGAG GMVHHRHRSS STRSGGGDLT LGLEPSEEEA PRSPLAPSEG

1090 1100 1110 1120 1130 1140
AGSDVFDGDL GMGAAKGLQS LPTHDPSPLQ RYSEDPTVPL PSETDGYVAP LTCSPQPEYV

1150 1160 1170 1180 1190 1200
NQPDVRPQPP SPREGPLPAA RPAGATLERP KTLSPGKNGV VKDVFAFGGA VENPEYLTPQ

1210 1220 1230 1240 1250
GGAAPQPHPP PAFSPAFDNL YYWDQDPPER GAPPSTFKGT PTAENPEYLG LDVPV

Fig. 3. The HER2 intracellular domain contains 3 putative CaM binding sites. The entire HER2 intracellular sequence was analyzed using the Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html). Three putative CaM binding domains were identified (bold print). The regions corresponding to HER2-N (red), HER2-M (underline) and HER2-C (blue) are shown.



interaction is validated by the absence of CaM from samples that were incubated with HER2-C or GST alone (Fig. 4B). The amount of HER2 in each sample was essentially identical (Fig. 4B). These data strongly suggest that residues 676–689 (KRRQQKIRKYTMRR) and 714–732 (ILKETELRKVKVLGSGAFG) of HER2 are necessary for its interaction with Ca^{2+}/CaM, while residues 676–689 of HER2 comprise the apocalmodulin binding region.

To confirm that the CaM-binding regions of HER2 we identified are sufficient to modulate its association with CaM, we created GST-fusion proteins of each site and evaluated the interaction with CaM. CaM was incubated with each peptide and GST-bound complexes were isolated, resolved by SDS-PAGE and processed by Western blotting. HER2-1 (residues 676–689) and HER2-2 (residues 714–732) bind essentially the same amount of Ca^{2+}/CaM, while no Ca^{2+}/CaM binds to HER2-3 (residues 883–902; Fig. 4C). The amount of Ca^{2+}/CaM bound to HER2-1,2 (residues 676–732) was substantially greater than that of either HER2-1 or HER2-2. HER2-1 and HER2-1,2 bind equivalent amounts of apocalmodulin, while no apocalmodulin binds to HER2-2 or HER2-3 (Fig. 4C). The specificity of the interaction is validated by the absence of CaM from samples that were incubated with GST alone. The amount of GST-fusion protein in each sample was essentially identical (Fig. 4C). These data indicate that residues 676–689 and 714–732 of HER2 are sufficient to mediate its interaction with Ca^{2+}/CaM, and imply a synergistic effect of the two binding regions. Residues 676–689 are sufficient for HER2-apocalmodulin binding.

3.3. CaM modulates HER2 phosphorylation

We employed a complimentary loss/gain of function strategy to ascertain the biological relevance of the CaM–HER2 interaction. For loss of function analysis, SkBR3 cells were treated with either vehicle or the specific cell-permeable CaM antagonist CGS9343B [32]. In parallel, HEK293 cells were transfected with vector (pcDNA3), HER2, or HER2 lacking the CaM binding regions. These constructs are termed HER2Δ1_{FL} (residues 676–689 deleted) and HER2Δ2_{FL} (residues 714–732 deleted). HER2 phosphorylation was evaluated by Western blotting. Inhibition of CaM function reduces HER2 phosphorylation in SkBR3 cells by ~70% (Fig. 5A; Fig. 5B, left panel). No significant

Fig. 4. Residues 676–689 and 714–732 of HER2 are necessary for CaM binding. A, Schematic representation of HER2 constructs depicting HER2 intracellular domain fragments and HER2 intracellular domain deletion mutants. The specific residues present in each fragment as well as those removed from each deletion mutant are indicated. HER2-N, N-terminal portion of HER2 intracellular domain (residues 676–966); HER2Δ1, N-terminal portion of HER2 intracellular domain with residues 676–689 deleted; HER2Δ2, N-terminal portion of HER2 intracellular domain with residues 714–732 deleted; HER2Δ3, N-terminal portion of HER2 intracellular domain with residues 883–902 deleted; HER2Δ1,3, N-terminal portion of HER2 intracellular domain with residues 676–689 and 883–902 deleted; HER2Δ1,2,3, N-terminal portion of HER2 intracellular domain with residues 676–689, 714–732 and 883–902 deleted; HER2-C, C-terminal portion of HER2 intracellular domain (residues 967–1255). B, Pure GST-HER2-N, GST-HER2Δ1, GST-HER2Δ2, GST-HER2Δ3, GST-HER2Δ1,3, GST-HER2Δ1,2,3, GST-HER2-C or GST alone was incubated with equal amounts of pure CaM in the presence (Ca^{2+}) or absence (EGTA) of Ca^{2+}. Complexes were isolated and washed as described in materials and methods and samples were resolved by SDS-PAGE. After running, the gel was cut at the 50 kDa marker. The bottom half was transferred to PVDF membrane and probed with anti-CaM antibody (Pulldown). The top half was stained with Coomassie to demonstrate essentially equal amounts of GST-HER2 in each sample (Coomassie). An aliquot of pure CaM (equivalent to 2% of the amount in each pulldown) was also resolved by SDS-PAGE and processed as described (Input). The data are representative of 5 independent experiments. C, Pure GST–HER2-1, GST–HER2-1,2, GST–HER2-2, GST–HER2-3 or GST alone was incubated with equal amounts of pure CaM in the presence (Ca^{2+}) or absence (EGTA) of Ca^{2+}. Complexes were isolated and washed as described in the materials and methods and samples were resolved by SDS-PAGE. After running, the gel was cut at the 20 kDa marker. The bottom half was transferred to PVDF membrane and probed with anti-CaM antibody (Pulldown). The top half was stained with Coomassie to demonstrate essentially equal amounts of peptide in each sample (Coomassie). An aliquot of pure CaM (equivalent to 2% of the amount in each pulldown) was also resolved by SDS-PAGE and processed as described (Input). The data are representative of 3 independent experiments.

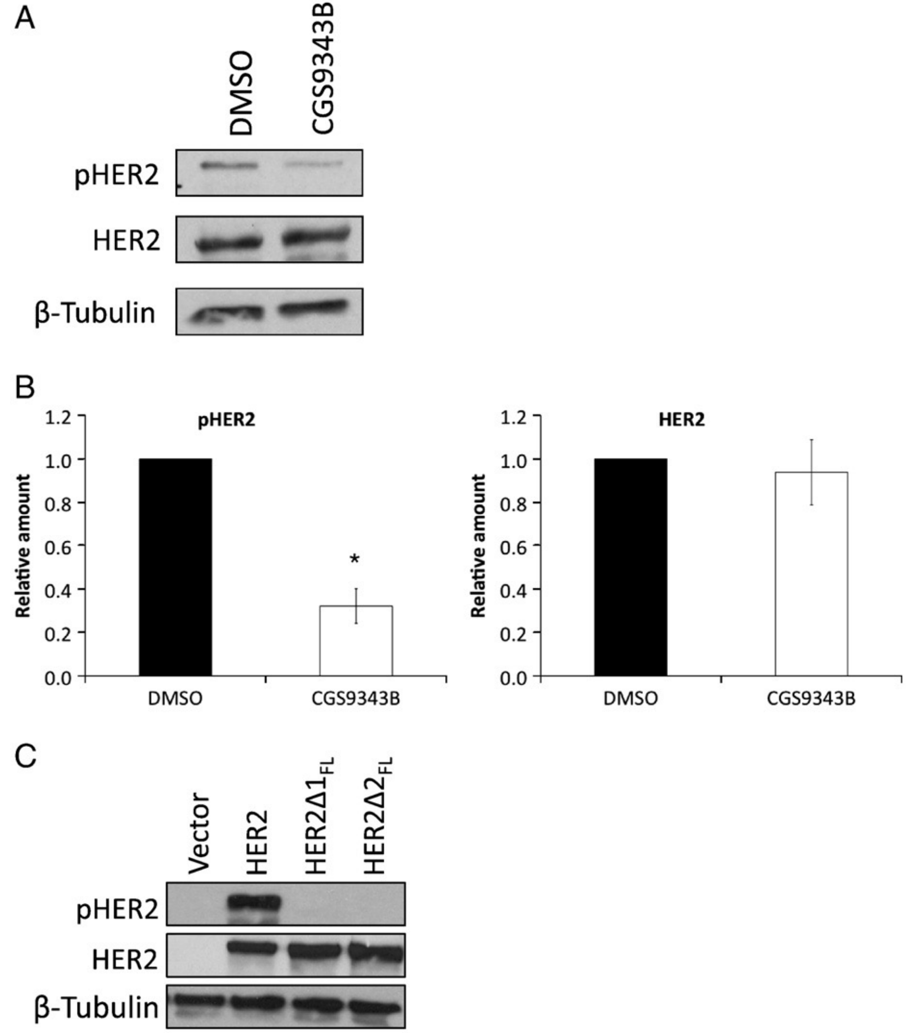


Fig. 5. CaM is necessary for HER2 phosphorylation. A, SkBR3 cells were serum-starved in the presence of vehicle (DMSO) or 10 μM CGS9343B for 48 h. Equal amounts of protein were resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-phospho-HER2, anti-HER2 and anti-β-Tubulin antibodies. The data are representative of 5 independent experiments. B, The amount of phospho-HER2 was quantified by densitometry and corrected for the amount of HER2 in the corresponding lysate. The amount of HER2 was quantified by densitometry and corrected for the amount of β-Tubulin in the corresponding lysate. The data, expressed relative to the amount of each protein in cells treated with DMSO (black bars), represent the mean ± SE ($n = 5$). $^*p < 0.05$. C, HEK293 cells were transfected with vector (pcDNA3), HER2, HER2Δ1_{FL} or HER2Δ2_{FL} prior to being serum-starved for 48 h. Equal amounts of protein were resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-phospho-HER2, anti-HER2 and anti-β-Tubulin antibodies. The data are representative of 3 independent experiments.

change in phospho-HER2 was observed when untreated cells were compared to cells treated with DMSO only (data not shown). Moreover, neither DMSO nor CGS9343B significantly reduces the total amount of HER2 (Fig. 5A; Fig. 5B, right panel) or β-Tubulin (Fig. 5A). In HEK293 cells, transfection of HER2 resulted in a clearly detectable phosphorylation band (Fig. 5C). By contrast, no phospho-HER2 was detected in cells transfected HER2Δ1_{FL} or HER2Δ2_{FL}. Note that the expression of HER2, HERΔ1_{FL} and HER2Δ2_{FL} was essentially identical (Fig. 5C). Together, these data indicate that CaM binding to HER2 is necessary for HER2 phosphorylation.

3.4. CaM binding to HER2 is necessary for HER2-stimulated cell growth

In HER2(+) breast tumors, aberrant HER2 phosphorylation stimulates cell cycle progression and uncontrolled cell proliferation [8]. To ascertain whether CaM modulates HER2 function, we measured HER2-stimulated cell growth under two different conditions. The CaM antagonist CGS9343B significantly inhibits proliferation of SkBR3 cells by ~30% (Fig. 6). Neither CGS9343B nor DMSO significantly affects cell viability (as determined by trypan blue exclusion; data not shown). CaM modulates multiple enzymes, several of which could be suppressed by CGS9343B treatment. Therefore, we tested the specific effect of CaM on HER2-stimulated cell growth by examining the effect of mutant HER2 constructs that lack binding to CaM. In HEK293 cells, transfection of HER2 increases cell growth by ~30% (Fig. 6B). In contrast, neither HER2Δ1_{FL} nor HER2Δ2_{FL} have any significant effect. These data indicate that CaM binding to HER2 is necessary for HER2 to stimulate cell growth.

4. Discussion

The functional interaction of CaM with EGFR has been the focus of investigations by several groups (reviewed in reference [33]). In contrast, despite the incontrovertible clinical importance of HER2, only one study has explored CaM–HER2 association [27]. Moreover, neither the region of HER2 with which CaM interacts nor the effect of CaM on HER2-stimulated tumorigenesis has been evaluated. Here, we show that CaM binds, in a Ca^{2+}-regulated manner, to two distinct sites on the N-terminal portion of the HER2 intracellular domain. Deletion of residues 676–689 and 714–732 from HER2 prevented CaM

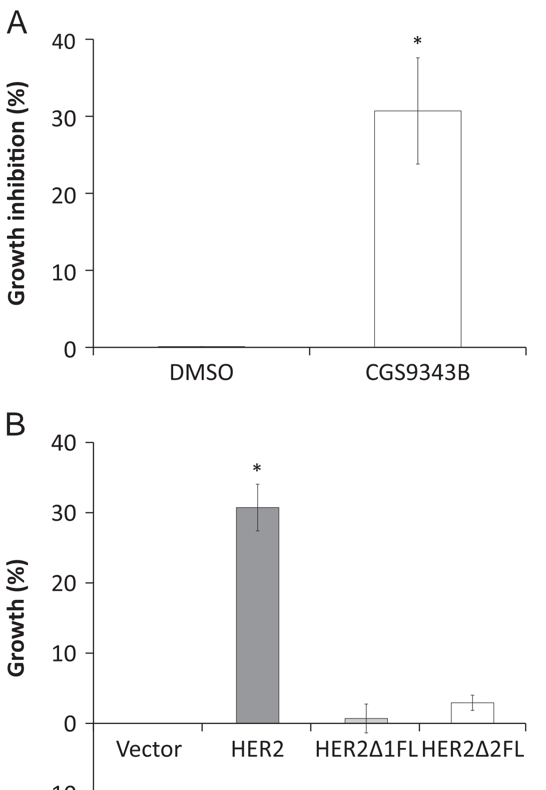


Fig. 6. CaM is necessary for HER2-stimulated cell growth. A, SkBR3 cells were serum-starved in the presence of vehicle (DMSO) or 10 μM CGS9343B for 48 h. Cell growth was measured using sulforhodamine B staining. The data, expressed relative to the number of cells in wells treated with DMSO (black bars), represent the mean ± SE (n = 5). *, $p < 0.05$. B, HEK293 cells were transfected with vector (pcDNA3), HER2, HER2Δ1_{FL} or HER2Δ2_{FL} prior to being serum-starved for 48 h. Cell growth was measured using sulforhodamine B staining. The data, expressed relative to the number of cells in wells transfected with vector (pcDNA3; black bars), represent the mean ± SE (n = 3). *, $p < 0.05$.

binding. Inhibition of CaM function or disruption of CaM–HER2 interaction reduced HER2 phosphorylation and HER2-stimulated cell growth. Collectively, these data suggest that manipulation of CaM–HER2 binding may be a feasible approach to treat patients with breast cancer.

Our data reveal that HER2 binds both Ca^{2+}/CaM and apocalmodulin. Although binding is considerably reduced in the absence of Ca^{2+}, analysis with both pure proteins and breast epithelial cell lysates consistently detected a specific interaction between apocalmodulin and HER2. These findings are in contrast to a prior report which observed an association between CaM and HER2 only in the presence of Ca^{2+} [27]. Several factors may account for these discrepant data. For example, while both studies evaluated binding of CaM immobilized on beads to HER2 in the SkBR3 cell line, different buffers were used to lyse the cells. Moreover, we examined binding of pure CaM and GST-HER2 to substantiate our CaM–Sepharose data. In contrast, the prior publication immunoprecipitated HER2 and used overlay with biotinylated CaM [27]. It is possible that the tertiary conformation adopted by HER2 immobilized on a membrane may sterically obscure the binding region for apocalmodulin, while permitting an association with Ca^{2+}/CaM.

The CaM binding regions of target proteins do not show strong sequence homology [34]. Nevertheless, two general characteristics may be observed. Many Ca^{2+}/CaM binding proteins have an amphipathic helix (comprising approximately 20 residues) that contains hydrophobic and basic amino acids [10]. Another CaM binding domain, termed the IQ motif, binds CaM in a predominantly Ca^{2+}-independent manner. The IQ motif generally comprises the sequence IQxxxRGxxxR (where x denotes any amino acid), however Ile^1 and Gly^7 are frequently not conserved [35]. Inspection of residues 676–689 of HER2 reveals a putative IQ motif: KRRQQKIRKYTMRR (the underlined residues comprise the IQ domain). Consistent with this *in silico* analysis, deletion of residues 676–689 prevents apocalmodulin from binding HER2. In contrast, deletion of the second putative CaM binding domain, residues 714–732, has no significant effect on apocalmodulin-HER2 association. These data are substantiated by our findings which reveal that residues 676–689 of HER2 are sufficient for apocalmodulin interaction, while residues 714–732 are unable to modulate apocalmodulin-HER2 binding. Together, these observations suggest that residues 676–689 represent the region of HER2 with which CaM interacts in a Ca^{2+}-independent manner.

Our findings with Ca^{2+}/CaM were different from those with apocalmodulin. Elimination of residues 676–689 from HER2 markedly attenuates, but does not abrogate, its interaction with Ca^{2+}/CaM, implying the existence of a second CaM binding region. Moreover, deletion of residues 714–732 (ILKETELRKVKVLGSGAFG) considerably reduces Ca^{2+}/CaM association with HER2, suggesting that this region is also necessary for Ca^{2+}/CaM interaction. Consistent with this observation, deletion of both regions 676–689 and 714–732 eliminates Ca^{2+}/CaM–HER2 binding. It is important to emphasize that we cannot exclude the possibility that the deletions we made alter the tertiary conformation of HER2. Nevertheless, our findings are supported by our analyses using synthesized peptides, which demonstrate that both residues 676–689 and residues 714–732 of HER2 interact with Ca^{2+}/CaM.

The interaction between CaM and EGFR has been characterized in some detail. CaM binds to the cytosolic juxtamembrane region of EGFR, amino acids 645–660 [23,36,37]. Sequence alignment reveals that this domain is similar to that of residues 676–689 of HER2 (Fig. 7). Although no other CaM binding region has been identified on EGFR, residues 714–732 of HER2 (the second CaM interacting region) exhibit 84% homology to the corresponding EGFR residues (Ile^{682}–Gly^{700}; Fig. 7). Unfortunately, no published study has analyzed the possible role of this domain in CaM–EGFR interaction. Each report that concluded that the juxtamembrane region of EGFR mediates its interaction with CaM analyzed only the juxtamembrane domain (which terminates at Phe^{688}) [23,36,37]. The region of EGFR corresponding to the second CaM binding domain of HER2 terminates at Gly^{700}. Several CaM targets, including other receptors, have more than one CaM binding site [38,39]. It therefore remains possible that CaM may associate with EGFR at a site distal to the juxtamembrane region.

ErbB receptor activity is regulated by autophosphorylation of tyrosine residues within the activation loop of the kinase domain [40,41], therefore HER2 catalytic activity may be measured by quantifying HER2 phosphorylation [42]. We report here that inhibition of CaM function with CGS9343B markedly reduces HER2

EGFR 645 -RRRHIVRKRTLRRLLQERELVEPLTPSGEAPNQALLRILKETEFKKIKVLGSGAFGTVY **703**
HER2 676 KRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGAFGTVY **735**

Fig. 7. Sequence alignment of the juxtamembrane portion of EGFR and HER2. Conserved residues are highlighted in yellow. The amino acids deleted from HER2Δ1 and HER2Δ2 are shown (bold print).

phosphorylation, suggesting that CaM is required for maximal HER2 activation. These data are supported by our observations which reveal that deletion of either CaM binding site from HER2 abrogates HER2 phosphorylation, and by a previous study which demonstrated that incubation of SkBR3 cells with the CaM antagonist W7 prevents HER2 phosphorylation in response to heregulin [27]. In HER2(+) breast neoplasms, aberrant HER2 phosphorylation stimulates uncontrolled cell proliferation and, consequently, rapid tumor growth [8]. Perhaps the most important finding to come from this study is, therefore, our observation that antagonism of CaM function or disruption of CaM–HER2 association significantly reduces HER2-stimulated cell growth. It is likely that this inhibition arises as a direct result of the effect of CaM on HER2 phosphorylation. *In vitro* studies have confirmed a direct link between decreased cell growth and reduced HER2 phosphorylation and signaling [43,44]. Furthermore, these reports have been validated in both animal models [45,46] and clinical investigations of primary human neoplasms [47,48].

In conclusion, we report both *in vitro* and in intact cells that CaM associates with HER2 in a Ca^{2+}-regulated, but not exclusively Ca^{2+}-dependent, manner. More importantly, we document a role for CaM in HER2 phosphorylation, and reveal a previously unrecognized function of CaM in HER2-stimulated tumorigenesis. When viewed in conjunction with prior observations that CaM antagonists augment anti-estrogen therapy [49], our findings have potential implications for the design of selectively targeted small molecule inhibitors for the treatment of breast cancer.

Conflicts of interest statement

The authors have nothing to disclose.

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References

[1] N.E. Hynes, H.A. Lane, ErbB receptors and cancer: the complexity of targeted inhibitors, Nat. Rev. Cancer 5 (2005) 341–354.
[2] Y. Yarden, M.X. Sliwkowski, Untangling the ErbB signalling network, Nat. Rev. Mol. Cell Biol. 2 (2001) 127–137.
[3] N.E. Hynes, G. MacDonald, ErbB receptors and signaling pathways in cancer, Curr. Opin. Cell Biol. 21 (2009) 177–184.
[4] R. Nahta, F.J. Esteva, Herceptin: mechanisms of action and resistance, Cancer Lett. 232 (2006) 123–138.
[5] D.J. Slamon, G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, W.L. McGuire, Human breast cancer: correlation of relapse and survival with amplification of the HER2/*Neu* oncogene, Science 235 (1987) 177–182.
[6] H.S. Cho, K. Mason, K.X. Ramyar, A.M. Stanley, S.B. Gabelli, D.W. Denney, D.J. Leahy, Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab, Nature 421 (2003) 756–760.
[7] T.P. Garrett, N.M. McKern, M. Lou, T.C. Elleman, T.E. Adams, G.O. Lovrecz, M. Kofler, R.N. Jorissen, E.C. Nice, A.W. Burgess, C.W. Ward, The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors, Mol. Cell 11 (2003) 495–505.
[8] M.M. Moasser, The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis, Oncogene 26 (2007) 6469–6487.
[9] D. Dankort, N. Jeyabalan, N. Jones, D.J. Dumont, W.J. Muller, Multiple ErbB2/*Neu* phosphorylation sites mediate transformation through distinct effector proteins, J. Biol. Chem. 276 (2001) 38921–38928.
[10] K.P. Hoeflich, M. Ikura, Calmodulin in action: diversity in target recognition and activation mechanisms, Cell 108 (2002) 739–742.
[11] L. Li, D.B. Sacks, Functional interactions between calmodulin and estrogen receptor α, Cell. Signal. 19 (2007) 439–443.
[12] C.B. Graves, R.R. Goewert, J.M. McDonald, The insulin receptor contains a calmodulin binding domain, Science 230 (1985) 827–829.
[13] D.B. Sacks, Y. Fujita-Yamaguchi, R.D. Gale, J.M. McDonald, Tyrosine-specific phosphorylation of calmodulin by the insulin receptor kinase purified from human placenta, Biochem. J. 263 (1989) 803–812.
[14] A. Bouhoute, G. Leclercq, Estradiol derivatives bearing the side chain of tamoxifen antagonize the association between the estrogen receptor and calmodulin, Biochem. Pharmacol. 47 (1994) 748–751.
[15] G. Castoria, A. Migliaccio, E. Nola, F. Auricchio, *In vitro* interaction of estradiol receptor with Ca^{2+}-calmodulin, Mol. Endocrinol. 2 (1988) 167–174.
[16] Z. Li, J.L. Joyal, D.B. Sacks, Calmodulin enhances the stability of the estrogen receptor, J. Biol. Chem. 276 (2001) 17354–17360.
[17] M.A. Schumacher, A.F. Rivard, H.P. Bachinger, J.P. Adelman, Structure of the gating domain of a Ca^{2+}-activated K^+ channel complexed with Ca^{2+}/calmodulin, Nature 410 (2001) 1120–1124.
[18] S.R. Johnston, New strategies in estrogen receptor positive breast cancer, Clin. Cancer Res. 16 (2010) 1979–1987.
[19] H. Brauch, V.C. Jordan, Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: the "personalised" approach? Eur. J. Cancer 45 (2009) 2274–2283.
[20] A. Bouhoute, G. Leclercq, Antagonistic effect of triphenylethylenic antiestrogens on the association of estrogen receptor to calmodulin, Biochem. Biophys. Res. Commun. 184 (1992) 1432–1440.
[21] E. San Jose, A. Benguria, P. Geller, A. Villalobo, Calmodulin inhibits the epidermal growth factor receptor tyrosine kinase, J. Biol. Chem. 267 (1992) 15237–15245.
[22] H. Li, A. Villalobo, Evidence for the direct interaction between calmodulin and the human epidermal growth factor receptor, Biochem. J. 362 (2002) 499–505.
[23] S. Aifa, K. Johansen, U.K. Nilsson, B. Liedberg, I. Lundstrom, S.P. Svensson, Interactions between the juxtamembrane domain of the EGFR and calmodulin measured by surface plasmon resonance, Cell. Signal. 14 (2002) 1005–1013.
[24] K. Yamane, C. Toyoshima, S. Nishimura, Ligand-induced functions of the epidermal growth factor receptor require the positively charged region asymmetrically distributed across plasma membrane, Biochem. Biophys. Res. Commun. 184 (1992) 1301–1310.
[25] H.M. Poppleton, H. Sun, J.B. Mullenix, G.J. Wiepz, P.J. Bertics, T.B. Patel, The juxtamembrane region of the epidermal growth factor receptor is required for phosphorylation of $G\alpha_s$, Arch. Biochem. Biophys. 383 (2000) 309–317.
[26] S. Aifa, J. Aydin, G. Nordvall, I. Lundstrom, S.P. Svensson, O. Hermanson, A basic peptide within the juxtamembrane region is required for EGF receptor dimerization, Exp. Cell Res. 302 (2005) 108–114.
[27] H. Li, J. Sanchez-Torres, A. Del Carpio, V. Salas, A. Villalobo, The ErbB2/*Neu*/HER2 receptor is a new calmodulin binding protein, Biochem. J. 381 (2004) 257–266.
[28] D.B. Sacks, S.E. Porter, J.H. Ladenson, J.M. McDonald, Monoclonal antibody to calmodulin: development, characterization and comparison with polyclonal anti-calmodulin antibodies, Anal. Biochem. 194 (1991) 369–377.
[29] Y.D. Ho, J.L. Joyal, Z. Li, D.B. Sacks, IQGAP1 integrates Ca^{2+}/calmodulin and Cdc42 signaling, J. Biol. Chem. 274 (1999) 464–470.
[30] Z. Li, D.B. Sacks, Elucidation of the interaction of calmodulin with the IQ motifs of IQGAP1, J. Biol. Chem. 278 (2003) 4347–4352.
[31] C.D. White, M. Coetsee, K. Morgan, C.A. Flanagan, R.P. Millar, Z.L. Lu, A crucial role for $G\alpha_{q/11}$, but not $G\alpha_{i/o}$ or $G\alpha_s$, in gonadotropin-releasing hormone receptor-mediated cell growth inhibition, Mol. Endocrinol. 22 (2008) 2520–2530.
[32] J.A. Norman, J. Ansell, G.A. Stone, L.P. Wennogle, J.W. Wasley, CGS9343B, a novel, potent, and selective inhibitor of calmodulin activity, Mol. Pharmacol. 31 (1987) 535–540.
[33] P. Sanchez-Gonzalez, K. Jellali, A. Villalobo, Calmodulin-mediated regulation of the epidermal growth factor receptor, FEBS J. 277 (2010) 327–342.
[34] A.R. Rhoads, F. Friedberg, Sequence motifs for calmodulin recognition, FASEB J. 11 (1997) 331–340.
[35] L.A. Jurado, P.S. Chockalingam, H.W. Jarrett, Apocalmodulin, Physiol. Rev. 79 (1999) 661–682.
[36] J. Martin-Nieto, A. Villalobo, The human epidermal growth factor receptor contains a juxtamembrane calmodulin binding site, Biochemistry 37 (1998) 227–236.
[37] S. Aifa, F. Frikha, N. Miled, K. Johansen, I. Lundstrom, S.P. Svensson, Phosphorylation of Thr^{654} but not Thr^{669} within the juxtamembrane domain of the EGF receptor inhibits calmodulin binding, Biochem. Biophys. Res. Commun. 347 (2006) 381–387.
[38] S. Patel, S.A. Morris, C.E. Adkins, G. O'Beirne, C.W. Taylor, Ca^{2+}-independent inhibition of inositol trisphosphate receptors by calmodulin: redistribution of calmodulin as a possible means of regulating Ca^{2+} mobilization, Proc. Natl Acad. Sci. USA 94 (1997) 11627–11632.
[39] W. Nunomura, Y. Takakuwa, M. Parra, J.G. Conboy, N. Mohandas, Ca^{2+}-dependent and Ca^{2+}-independent calmodulin binding sites in erythrocyte protein 4.1. Implications for regulation of protein 4.1 interactions with transmembrane proteins, J. Biol. Chem. 275 (2000) 6360–6367.
[40] M. Huse, J. Kuriyan, The conformational plasticity of protein kinases, Cell 109 (2002) 275–282.
[41] J.A. Adams, Activation loop phosphorylation and catalysis in protein kinases: is there functional evidence for the autoinhibitor model? Biochemistry 42 (2003) 601–607.
[42] R. Bose, H. Molina, A.S. Patterson, J.K. Bitok, B. Periaswamy, J.S. Bader, A. Pandey, P.A. Cole, Phosphoproteomic analysis of HER2/*Neu* signaling and inhibition, Proc. Natl Acad. Sci. USA 103 (2006) 9773–9778.
[43] D.L. Shattuck, J.K. Miller, K.L. Carraway, C. Sweeney, Met receptor contributes to trastuzumab resistance of HER2-overexpressing breast cancer cells, Cancer Res. 68 (2008) 1471–1477.
[44] M.M. Moasser, A. Basso, S.D. Averbuch, N. Rosen, The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells, Cancer Res. 61 (2001) 7184–7188.
[45] J.P. Delord, C. Allal, M. Canal, E. Mery, P. Rochaix, I. Hennebelle, A. Pradines, E. Chatelut, R. Bugat, S. Guichard, P. Canal, Selective inhibition of HER2 inhibits AKT

signal transduction and prolongs disease free survival in a micrometastasis model of ovarian carcinoma, Ann. Oncol. 16 (2005) 1889–1897.
[46] M.P. Piechocki, S.K. Dibbley, F. Lonardo, G.H. Yoo, Gefitinib prevents cancer progression in mice expressing the activated rat HER2/*Neu*, Int. J. Cancer 122 (2008) 1722–1729.
[47] E. Tokunaga, Y. Kimura, E. Oki, N. Ueda, M. Futatsugi, K. Mashino, M. Yamamoto, M. Ikebe, Y. Kakeji, H. Baba, Y. Maehara, Akt is frequently activated in HER2/*Neu*-positive breast cancers and associated with poor prognosis among hormone-treated patients, Int. J. Cancer 118 (2006) 284–289.
[48] S.S. Park, S.W. Kim, Activated Akt signaling pathway in invasive ductal carcinoma of the breast: correlation with HER2 overexpression, Oncol. Rep. 18 (2007) 139–143.
[49] O.S. Frankfurt, E.V. Sugarbaker, J.A. Robb, L. Villa, Synergistic induction of apoptosis in breast cancer cells by tamoxifen and calmodulin inhibitors, Cancer Lett. 97 (1995) 149–154.