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The antibody sc-33040-R fails to specifically recognize phosphorylation of ErbB4 on tyrosine1056

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Abstract

Phosphorylation state specific antibodies are important reagents for characterizing protein phosphorylation and signaling. However, these antibodies require proper validation to determine that they do not cross-react with the unphosphorylated peptide or with other phosphoproteins. We have previously shown that phosphorylation of tyrosine1056 of ErbB4 is critical for it to inhibit colony formation on plastic by human tumor cell lines. Thus, an antibody directed against this site would be useful for studying ErbB4 signaling and coupling to biological responses. Here, we demonstrate that a commercially available antibody raised against a phosphopeptide corresponding to the carboxyl-terminal domain of the ErbB4 receptor tyrosine kinase fails to exhibit appropriate specificity. Thus, this antibody does not appear to be suitable for studying ErbB4 phosphorylation or signaling.

Keywords

ErbB4; receptor tyrosine kinase; tyrosine phosphorylation; phosphospecific antibody

Introduction

ErbB4 (HER4) is a member of the epidermal growth factor receptor (EGFR/ErbB) family of receptor tyrosine kinases, a family that also includes EGFR/ErbB1/HER1, ErbB2/HER2/Neu and ErbB3/HER3 (Yarden and Sliwkowski 2001). Signaling by these receptors is characterized by phosphorylation of their cytoplasmic tyrosine residues, which permits coupling to downstream effectors and biological responses (Yarden and Sliwkowski 2001). Indeed, increased phosphorylation of EGFR and ErbB2 is a hallmark of many human tumors and antibodies that recognize phosphorylated EGFR or ErbB2 have been used to monitor signaling by these receptors in tumor specimens and cell lines (Bangalore et al. 1992; Epstein et al. 1992; DiGiovanna and Stern 1995; DiGiovanna et al. 1996; Ouyang et al. 1996; DiGiovanna 1997; Ouyang et al. 1999; Thor et al. 2000; Ouyang et al. 2001; Onguru et al. 2004; Modi et al. 2005; Thelemann et al. 2005; Van Schaeysbroeck et al. 2005; Mulloy et al. 2007; Rittie et al. 2007). However, to be useful tools, these antibodies need to be properly validated to assure specificity for the expected target epitope.

The roles that ErbB4 plays in human malignancies have yet to be definitively established. ErbB4 over expression is observed in human tumor samples and ErbB4 expression is required for the stimulation of cell proliferation by members of the EGF family of polypeptide hormones (Kumar and Vaddlamudi 2000; Yarden and Sliwkowski 2001). These data suggest that ErbB4 may, like EGFR and ErbB2, behave as an oncogene. In contrast, ErbB4 expression in many

tumors is reduced relative to that observed in the normal epithelia (Robinson et al. 1996; Grasso et al. 1997; Lyne et al. 1997; Witton et al. 2003; Tovey et al. 2004). Moreover, ErbB4 expression in breast tumor samples correlates with a more favorable prognosis (Kew et al. 2000; Suo et al. 2002; Witton et al. 2003; Tovey et al. 2004; Junttila et al. 2005). Finally, constitutive ErbB4 signaling is coupled to apoptosis and/or growth arrest in human prostate, breast and pancreatic tumor cell lines (Williams et al. 2003; Gallo et al. 2006; Pitfield et al. 2006). Collectively, these data indicate that ErbB4 behaves as a tumor suppressor in some contexts.

One of the factors that has confounded efforts to determine the roles that ErbB4 plays in human malignancies is the existence of several ErbB4 transcriptional splicing isoforms. In particular, the Cyt-2 (CT-b) isoforms lack sixteen amino acids (Ser1046 through Gly1061) present in the canonical Cyt-1 (CT-a) isoforms (Elenius et al. 1999). The Cyt-2 isoforms lack the tumor suppressor activity displayed by the Cyt-1 isoforms (Gallo et al. 2006). Indeed, phosphorylation of a tyrosine residue (Tyr1056) absent in the Cyt-2 isoforms appears to be critical for ErbB4 tumor suppressor activity (Gallo et al. 2006; Pitfield et al. 2006). Thus, measuring the phosphorylation of ErbB4 at Tyr1056 may be a strategy for monitoring ErbB4 tumor suppressor activity in tumor samples and cell lines and may help to resolve the roles that ErbB4 signaling plays in human malignancies. Recently, Santa Cruz Biotechnology began marketing rabbit polyclonal antibody sc-33040-R. This antibody is raised against a short peptide that encompasses Tyr1056 and is phosphorylated on that tyrosine residue. Here, we describe our efforts to determine the specificity of this reagent.

Materials and methods

We have previously demonstrated that ψ 2 fibroblasts that ectopically express the constitutively active Q646C mutant of ErbB4 display ErbB4 phosphorylation on Tyr1056 (Gallo et al. 2006). Thus, we used ErbB4 immunoprecipitated from a lysate of this cell line to analyze ErbB4 phosphorylation on Tyr1056 using sc-33040-R. As controls, we used ErbB4 immunoprecipitated from lysates of ψ 2 cells that express the ErbB4 Q646C Kin⁻ mutant, which lacks kinase activity; the ErbB4 Q646C Chg1 (Y1056F) mutant, in which Tyr1056 is replaced by a phenylalanine residue; and ErbB4 Q646C CT-b, which lacks Ser1046 through Gly1061 (Gallo et al. 2006). As additional controls, lysates of ψ 2 fibroblasts (Mann et al. 1983) that had been transfected with pLXSN EGFR (Riese et al. 1995a) and C127 fibroblasts that endogenously express platelet-derived growth factor (PDGF) receptor (Riese et al. 1995b) were employed. These cell lines display minimal endogenous ErbB4 expression (Zhang et al. 1996). Prior to lysis, these cell lines were either mock stimulated with phosphate buffered normal saline (PBS) or stimulated with 100 ng/ml EGF or 100 ng/ml PDGF-BB, respectively.

Lysates were prepared and ErbB4 was immunoprecipitated from the ψ 2/ErbB4 cell lines using a specific anti-ErbB4 antibody (Santa Cruz Biotechnology SC-283) as described elsewhere (Penington et al. 2002; Williams et al. 2003). These immunoprecipitates and lysates from the ψ 2/EGFR cells and the C127 cells were divided in half and resolved by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) using two gels. Varying amounts of ErbB4 immunoprecipitated from ψ /ErbB4 Q646C cells were resolved in order to construct phosphorylation and expression calibration curves. Samples were electroblotted onto two polyvinylidene fluoride membranes. One blot was probed with a generic anti-phosphotyrosine antibody (4G10, Upstate Bio-technology) and the other with the anti-phospho-Tyr1056 (sc-33040-R, lot J1005, Santa Cruz Biotechnology). The anti-phosphotyrosine immunoblotting was performed as described previously (Penington et al. 2002; Williams et al. 2003).

The anti-phosphoTyr1056 immunoblotting was performed according to the protocol recommended by the manufacturer. Briefly, the membrane was blocked by incubation for 45 min at room temperature in 1% bovine serum albumin (BSA) and 1% milk in Tris-buffered normal saline supplemented with 0.05% Tween-20 (TBST). The blot was then incubated for 1 h at room temperature in the anti-phosphoTyr1056 antibody diluted 1:500 in 1% BSA and 1% milk in TBST. The blot was briefly rinsed once in 100 ml TBST and was then washed three times in 100 ml TBST for 5 min each at room temperature with shaking. The blot was incubated for 45 min at room temperature in a goat anti-rabbit HRP-conjugated antibody (Pierce) diluted 1:2000 in 5% milk in TBST. The blot was briefly rinsed once in 100 ml TBST, was washed three times in 100 ml TBST for 5 min each, and was washed once for 5 min in 100 ml of TBST. Primary antibody binding was visualized by enhanced chemiluminescence (ECL—Amersham). The antibodies bound to the anti-phosphotyrosine and anti-phosphoTyr1056 immunoblots were stripped by incubation for 10 min at 80°C in 10 ml of stripping buffer (200 mM glycine, pH 2.5 and 0.05% Tween 20; Jolival et al. 2006). Membranes were rinsed in TBST and blotted with an anti-ErbB4 antibody (SC-283 Santa Cruz Biotechnology) as described above.

Results

As seen in Figure 1A (upper panel), EGF and PDGF stimulate tyrosine phosphorylation of a protein of 180 kDa apparent molecular weight in the ψ 2 EGFR and C127 cells, respectively. Because corresponding bands are not observed in the ErbB4 immunoblot (lower panel), it is presumed that these bands represent tyrosine phosphorylated EGFR and PDGF receptor. The constitutively active ErbB4 Q646C mutant is abundantly tyrosine phosphorylated, as is the ErbB4 Q646C CT-b mutant. The ErbB4 Q646C Chg1 mutant displays a lesser but nonetheless detectable amount of phosphorylation, but the ErbB4 Q646C Kin- mutant fails to display detectable phosphorylation. These data are consistent with our previous published results (Gallo et al. 2006; Pitfield et al. 2006).

As seen in Figure 1B (upper panel), the anti-phosphoTyr1056 antibody displays immunoreactivity that is very similar to that displayed by the generic 4G10 anti-phosphotyrosine antibody. The sc-33040-R anti-phosphoTyr1056 antibody reacts with the ErbB4 Q646C positive control and exhibits minimal reactivity with the ErbB4 Q646C Kin⁻ negative control. However, the antibody appears to react strongly with phosphorylated EGFR and less so with phosphorylated PDGF receptor. The antibody reacts strongly with the ErbB4 Q646C CT-b mutant and less strongly with the ErbB4 Q646C Chg1 (Y1056F) mutant, both of which lack Tyr1056. These data indicate that the sc-33040-R antibody is not specific for phosphorylated ErbB4 and is not specific for phosphotyrosine 1056 of ErbB4.

Discussion

Here, we show that the sc-33040-R antibody fails to specifically recognize ErbB4 that is phosphorylated on tyrosine1056. The results of our lab and of others suggest that an antibody with that specificity could be a very useful reagent for assessing the signaling and biological activity of ErbB4. Such a reagent would be useful in our studies of the inhibition of human tumor cell growth by ErbB4 (Gallo et al. 2006; Pitfield et al. 2006). Additionally, since this site of phosphorylation differs between the two ErbB4 carboxyl-terminal splicing isoforms, antibodies that recognize the phosphorylated and unphosphorylated forms of this epitope could be useful in evaluating isoform-specific expression and signaling. This is especially important because of the recent work suggesting that the different ErbB4 isoforms have distinct biological functions (Elenius et al. 1999; Junttila et al. 2000; Maatta et al. 2006).

Emerging evidence indicates that the various ErbB4 ligands are functionally distinct. It has been proposed that these ligands stimulate phosphorylation at distinct sets of ErbB4 tyrosine residues (Sweeney et al. 2000; Hobbs et al. 2002; Hobbs et al. 2004). Reagents that can detect site-specific ErbB4 phosphorylation could be useful in evaluating this hypothesis and could possibly enable one to predict the biological activity of an ErbB4 ligand based on the pattern of ErbB4 tyrosine phosphorylation that it stimulates. However, the data presented here suggest that sc-33040-R fails to exhibit the specificity required for these analyses. Furthermore, this report should serve as notice that analogous antibodies should be carefully and comprehensively validated before they are used for such purposes.

Currently, there is one other commercially available ErbB4 phosphorylation state specific antibody. This rabbit monoclonal antibody (21A9) from Cell Signaling Technology is directed against phosphotyrosine 1284 of ErbB4. Based on the results shown in the product data sheet, this antibody does not appear to cross-react with other tyrosine phosphorylated ErbB receptors or with other tyrosine phosphoproteins. However, additional testing of this reagent will be required to demonstrate site specificity. Furthermore, antibodies specific for the other sites of ErbB4 phosphorylation are needed before one will be able to use these antibodies to map and compare the sites of ErbB4 tyrosine phosphorylation. Given the value of this approach in characterizing EGFR signaling, it appears that such a panel of phosphorylation state specific ErbB4 antibodies would be extremely useful.

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Figure 1.

sc-33040-R is not specific for tyrosine phosphorylation of ErbB4 at Y1056. Western blots were generated using whole cell lysates and immunoprecipitations from cells expressing the indicated receptors. Cells expressing EGFR or PDGFR were stimulated with an appropriate ligand to induce tyrosine phosphorylation. Constitutively active ErbB4 mutants that display phosphorylation of Y1056 (ErbB4 Q646C) or lack it (ErbB4 Q646C Chg1, ErbB4 Q646C Kin⁻, and ErbB4 Q646C CT-b) were used. Blots were stripped and reprobed using an anti-ErbB4 antibody. The indicated molecular weight marker is approximately 203 kDa. A. Immunoblot generated using a generic anti-phosphotyrosine monoclonal antibody (4G10). B. Immunoblot generated using the anti-phospho ErbB4 Y1056 polyclonal antiserum.