BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **221,** 140–145 (1996)
ARTICLE NO. 0559

p66^{Shc} Isoform Down-Regulated and Not Required for HER-2/neu Signaling Pathway in Human Breast Cancer Cell Lines with *HER-2/neu* Overexpression

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Received February 27, 1996

The *HER2/neu* protooncogene encodes a transmembrane receptor tyrosine kinase of M_r 185 kDa (called p185) which is structurally and functionally homologous to the epidermal growth factor receptor. Shc proteins are important downstream signal transducers of receptor tyrosine kinases. We reported here a novel finding that p66^{Shc} was absent or nearly absent in p185-overexpressing breast cancer cells. This inverse correlation of p185 overexpression and p66^{Shc} expression is probably specific to breast cancer cells because this phenomenon was not observed in p185-overexpressing human ovarian, lung, or oral cancer cells, or mouse fibroblast cells. In contrast, the p52^{Shc} and p46^{Shc} isoforms were expressed at similar levels in both p185-overexpressing and p185 basal level breast cancer cell lines. Furthermore, tyrosine phosphorylation of p52^{Shc} and p46^{Shc} and subsequent formation of Shc/Grb2 complex were detected in breast cancer cells in which the p185 tyrosine kinase is activated, indicating that p66^{Shc} is not required for mediating the HER-2/neu signaling pathway in breast cancer cells.

The *HER2/neu* (also known as *erbB*-2) protooncogene encodes an M_r 185,000 transmembrane glycoprotein (called p185) with intrinsic tyrosine kinase activity homologous to the epidermal growth factor (EGF) receptor (1–4). It is well known that amplification and/or overexpression of *HER2/neu* correlates to approximately 30% of human breast cancer (5) and that aberrant activation of its tyrosine kinase confers transforming ability on p185 (6). However, the downstream signaling pathways mediating the oncogenic signal of p185 in breast cancer are not well understood. Our recent studies have demonstrated that tyrosine phosphorylation of Shc, termed for *S*rc *h*omology 2α-*c*ollagen-related (7), and subsequent formation of the Shc/Grb2/Sos complex play a major role in cell transformation mediated by the point mutation-activated p185 in mouse fibroblast NIH3T3 cells (8, 9). These observations suggest that Shc proteins may play an important role in transmitting the oncogenic signal of p185 in breast cancer. The mammalian *Shc* locus encodes three overlapping isoforms of 46, 52, and 66 kDa (p46^{Shc}, p52^{Shc}, and p66^{Shc}) (7). p46^{Shc} and p52^{Shc} arise from the use of alternative translation initiation sites within the same transcript. p46^{Shc} is an amino-terminal 59-amino acid truncation of p52^{Shc}. In contrast, p66^{Shc} most likely arises from an alternatively spliced transcript, since there is only *Shc* gene and the antibodies specific to the carboxyl-terminus cross-react with all three Shc isoforms (7). Although the importance of Shc in signal transduction of receptor tyrosine kinases has been extensively investigated, the functional differences of the three isoforms remain unclear. To study the functional role of Shc in breast cancer cells that overexpress *HER2/neu*, we first compared the expression levels of Shc proteins in 10 different breast cancer cell lines that overexpress or have basal level expression of *HER2/neu*. To our surprise, we found that no or a very little amount of p66^{Shc} was detected in the six p185-overexpressing cell lines, whereas significant amounts of p66^{Shc} existed in the other four cell lines that expressed p185 at basal levels. In contrast, the levels of p46^{Shc} and p52^{Shc} expression were comparable in each of the 10 cell lines. It is interesting that the inverse correlation of expression

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0006-291X/96 $18.00

of p185 and $p66^{Shc}$ appeared to be cell type specific, since $p66^{Shc}$ was detected in human ovarian, lung, and oral cancer cells and in mouse fibroblast cells irrespective of the p185 expression level. We demonstrated, furthermore, that $p66^{Shc}$ was not required for mediating the oncogenic signal of HER-2/neu to downstream signaling pathways in human breast cancer cells.

METHODS

Cell lines and culture. The human cancer cell lines used in this study were from ATCC (American Type Culture Collection, Rockville, MD). DHFR/G8 cells were derived from NIH3T3 cells expressing the transfected rat *HER2/neu* gene (3). Cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal calf serum under an atmosphere of 5% CO_2 at 37°C.

Antibodies. The monoclonal antibody c-neu-Ab3 targeting the carboxyl-terminal domain of p185 was obtained from Oncogene Science. The recombinant antiphosphotyrosine antibody (RC20), the monoclonal anti-Grb2 antibody, and the rabbit anti-Shc polyclonal antibody were purchased from Transduction Laboratories.

Immunoprecipitation and Western blotting. Cells were harvested at subconfluence and subjected to lysis in 1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, 10 μg/ml leupeptin, and 5 μg/ml aprotinin. Protein concentrations were determined against standardized controls using the Bio-Rad protein assay kit. Lysates were immunoprecipitated with appropriate antibodies according to the suppliers' specifications and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filters (Schleicher & Schuell). Filters were blocked with 5% dry milk powder in TPBS (0.05% Tween 20 in PBS), incubated with appropriate antibodies, incubated with goat anti-mouse or goat anti-rabbit IgG-HRP (Boehringer-Mannheim) and visualized by the ECL chemiluminescence system.

Stripping of Western blots. Used immunoblots were stripped by incubation with 62.5 mM Tris.Cl (pH 6.8), 2% SDS and 100 mM β-mercaptoethanol at 75°C for 30 min. Filters were then washed twice with TPBS and reprobed with other antibodies.

RESULTS AND DISCUSSION

To study the functional role of Shc proteins in *HER2/neu*-overexpressing breast cancer cells, we set out to evaluate the expression levels of Shc isoforms in 10 different breast cancer cell lines that express *HER2/neu* at various levels (10, 11). We first confirmed the p185 levels in these cells by immunoblot analysis with the monoclonal anti-p185 antibody. As shown in figure 1 (top panel), the p185 levels were readily detectable in the six *HER2/neu*-overexpressing cell lines, while under the same conditions the other four cell lines had no detectable p185. The p185 protein levels in these cell lines were generally consistent to previously described data on *HER2/neu* mRNA expression levels, with one exception. Previous data showed the *HER2/neu* mRNA levels in the SKBR-3 and

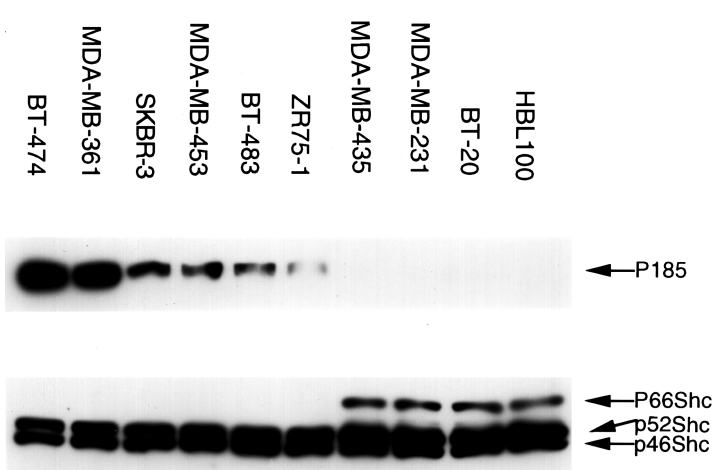

FIG. 1. Inverse correlation of expression of p185 and $p66^{Shc}$ in breast cancer cells. Fifty micrograms of cell extracts from various cell lines as indicated were subject to 6% SDS/PAGE. After transfer, the top portion of the nitrocellulose filter was probed with c-neu-mAb3, a monoclonal anti-p185 antibody, the lower part was incubated with polyclonal anti-Shc antibody.

MDA-MB-361 cell lines to be 128-fold and 64-fold over that of HBL100, respectively. Our western blot data indicated that the p185 protein level in MDA-MB-361 was higher than that in SKBR-3. This discrepancy may be due to heterogeneity in human breast cancer cells (12).

We compared the Shc protein levels in these cells with the anti-Shc polyclonal antibody (Fig. 1, bottom panel). To our surprise, the $p66^{Shc}$ isoform was clearly detected only in the four p185 basal level cell lines. No appreciable amounts of $p66^{Shc}$ were observed in any of the p185-overexpressing cell lines. Only a very faint band corresponding to the position of $p66^{Shc}$ was detected in the lanes of SKBR3 and MDA-MB-361 after longer exposure (data not shown). In contrast, the $p52^{Shc}$ and $p46^{Shc}$ protein levels were comparable in each of the 10 cell lines. These observations suggest an inverse correlation of expression of p185 and $p66^{Shc}$ in breast cancer cells.

To determine whether this inverse correlation of expression of p185 and $p66^{Shc}$ exists in other cell types, we examined by western analysis the $p66^{Shc}$ protein levels in human ovarian cancer cells and mouse fibroblast cells that overexpress *HER2/neu.* As shown in figure 2 (top panel), human ovarian cancer cell lines OVCAR3 and SKOV3 overexpress p185, while human ovarian cancer cell line 2774 does not express p185. DHFR/G8 is a NIH3T3-derived cell line overexpressing the rat *HER2/neu* gene (3). Down-regulation of $p66^{Shc}$ was not observed in cell lines OVCAR3, SKOV3, or DHFR/G8 cells (Fig. 2, bottom panel). The extra faint band on the top of $p52^{Shc}$ in some lanes was not always detectable in separate experiments. It is not yet clear whether this extra band represents an unidentified minor isoform of Shc. Since these cell lines have p185 levels higher than or similar to those of breast cancer cells tested in this study (Fig. 2 and data not shown), the appearance of $p66^{Shc}$ in these cell lines indicated that the inverse correlation of expression of p185 and $p66^{Shc}$ is not ubiquitous but cell type specific. Furthermore, we did not detect this inverse correlation in p185-overexpressing human oral cancer cell lines (13) or in stable transfectants derived from human lung cancer cells expressing *HER2/neu* cDNA (14) (data not shown). Taken together, these data suggest that the inverse correlation of p185 overexpression and $p66^{Shc}$ expression may be limited to human breast cancer cells.

To date the functional differences of the three Shc isoforms have not been investigated, probably because of an inability to clone the $p66^{Shc}$ cDNA. Our previous data have shown that the activated p185 can induce tyrosine phosphorylation of all three Shc isoforms and subsequent Shc/Grb2

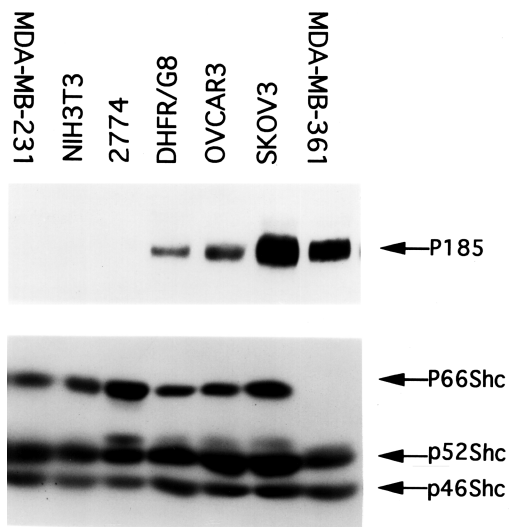

FIG. 2. Down-regulation of $p66^{Shc}$ is cell type specific. Fifty micrograms of cell lysates from different cell lines as indicated were subject to 6% SDS/PAGE. After transfer, the top portion of the nitrocellulose filter was probed with c-neu-mAb3, the lower part was incubated with polyclonal anti-Shc antibody.

complex formation and further downstream signaling pathways in fibroblast cells (8). It is also known that the p185 tyrosine kinase is activated in p185-overexpressing cells (15, 16). The absence of $p66^{Shc}$ in p185-overexpressing breast cancer cells therefore led us to examine whether the $p52^{Shc}$ and $p46^{Shc}$ isoforms can be tyrosine phosphorylated by the activated p185 in these cells and whether, in the absence of $p66^{Shc}$, they can form complex with Grb2 to mediate downstream signal pathways. We used the anti-Shc polyclonal antibody to immunoprecipitate Shc and associated proteins from two p185-overexpressing cell lines, SKBR3 and MDA-MB-453, and two p185 basal level control lines, MDA-MB-231 and MDA-MB-435. After immunoprecipitation, the immunocomplexes were separated by SDS/PAGE and subjected to western analysis with anti-p185 (Fig. 3a), recombinant anti-phosphotyrosine antibody (RC20) (Fig. 3b), or monoclonal anti-Grb2 antibody (Fig. 3c). As shown in figure 3a, Shc proteins were physically associated with the activated p185 in SKBR3 and MDA-MB-453 cells, which is similar to our previous data showing the complex formation of p185 and Shc proteins in mouse fibroblast cells expressing the point mutation-activated *HER2/neu* (8). Both $p52^{Shc}$ and $p46^{Shc}$ were tyrosine phosphorylated in SKBR3 and MDA-MB-453 cells, while no appreciable amounts of tyrosine phosphorylated Shc proteins were detected in either MDA-MB-231 or MDA-MB-435 cells. Furthermore, the Shc/Grb2 complex formed in SKBR3 and MDA-MB-453 cells but not in the two control cell lines. To show equal loading, we stripped the filter (used in panel b) and re-probed it with the anti-Shc polyclonal antibody. As shown in figure 3d, equal amounts of various cell extracts were applied in the immunoprecipitation. This experiment again demonstrated that $p66^{Shc}$ was undetectable in either SKBR3 or MDA-MB-453 cells. A recent report already showed that the downstream MAP kinase signaling pathway is activated in *HER-2/neu*-overexpressing human breast cancer cells including SKBR3 and MDA-MB-453 (17). Taken together, these observations suggest that a lack of $p66^{Shc}$

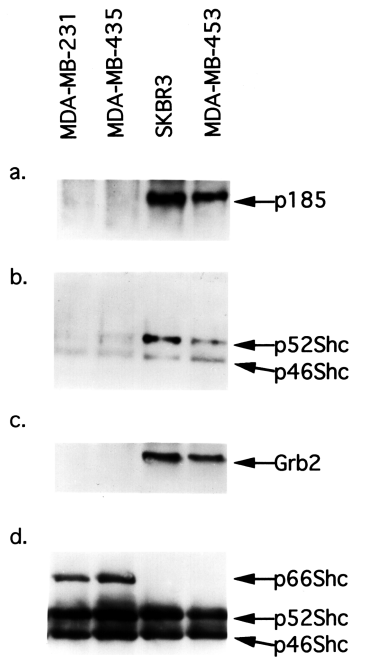

FIG. 3. Tyrosine phosphorylation of $p52^{Shc}$ and $p46^{Shc}$ by the activated p185 in breast cancer cells. Two milligrams of lysates from each cell line were immunoprecipitated with polyclonal anti-Shc antibody. Immunocomplexes were dissected by 10% SDS/PAGE, followed by immunoblotting analysis with either c-neu-Ab3 (panel a), recombinant anti-phosphotyrosine antibody (panel b), or monoclonal anti-Grb2 antibody (panel c). The filter used in panel b was stripped off and re-probed with polyclonal anti-Shc antibody (panel d). Equal loading was confirmed in panel d.

does not affect tyrosine phosphorylation of $p52^{Shc}$ and $p46^{Shc}$, Shc/Grb2 complex formation, or the downstream MAP kinase signaling pathway induction by the activated p185 in breast cancer cells. In other words, $p66^{Shc}$ is not required for mediating the oncogenic signal of p185 in human breast cancer cells that overexpress *HER-2/neu.*

It is generally believed that Shc proteins are important signal mediators for receptor tyrosine kinases. However, little is known about the distinct function of any of the three Shc isoforms. All three isoforms appear to bind and be tyrosine phosphorylated by the activated p185 and EGF receptor in fibroblast cells (7–9). A recent report demonstrated that the major insulin-dependent Shc signaling pathway occurred through the $p52^{Shc}$ isoform, whereas the EGF receptor displayed similar substrate specificity for both the $p52^{Shc}$ and the $p46^{Shc}$ forms (18). This suggests that preferential tyrosine phosphorylation of Shc isoforms may underlie physiological specificity in the signaling pathways of different receptor tyrosine kinases. Differential physiological specificity among the Shc isoforms is further supported by the fact that $p52^{Shc}$ and $p66^{Shc}$, but not $p46^{Shc}$, are associated with the PEST tyrosine phosphatase (19,20). This interaction can be enhanced by protein kinase C activator phorbol 12-myristate 13-acetate and G protein receptor agonist, but not by EGF or serum, suggesting cross-talk between the G protein receptor and the Shc signaling pathway. In addition, $p66^{Shc}$ was not expressed in any human hematopoietic cell lines tested (7), which is similar to the situation in *HER2/neu*-overexpressing breast cancer cells described in this study. It is unclear whether a common mechanism leads to the absence of $p66^{Shc}$ in hematopoietic cell lines and p185-overexpressing breast cancer cell lines, but our data suggest that both cell type specificity and overexpression of p185 may contribute to the down-regulation of $p66^{Shc}$.

We reported here a novel finding that $p66^{Shc}$ was down-regulated in p185-overexpressing breast cancer cells. This inverse correlation of p185 overexpression and $p66^{Shc}$ expression is probably specific to breast cancer cells because this phenomenon was not observed in p185-overexpressing mouse fibroblasts or human ovarian, oral, or lung cancer cells. In contrast, the $p52^{Shc}$ and $p46^{Shc}$ isoforms were expressed at similar levels in p185-overexpressing breast cancer cell lines as in those expressing basal levels of p185. Moreover, $p52^{Shc}$ and $p46^{Shc}$ without $p66^{Shc}$ appear to be sufficient to transmit the oncogenic signal of the activated p185 in breast cancer cells. Further study of the relationship between $p66^{Shc}$ expression and p185 overexpression in breast cancer cells may help us understand differential physiological functions among the Shc isoforms.

ACKNOWLEDGMENTS

This work was supported by NIH grants (CA58880 and CA60856), US Army Breast Cancer Grant (DAMD 17-94-J-4315) and Breast Cancer Basic Research Program of M. D. Anderson Cancer Center to M.C.H and a Rosalie B. Hite predoctoral fellowship to Y.X.

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