The Phosphoprotein StarD10 Is Overexpressed in Breast Cancer and Cooperates with ErbB Receptors in Cellular Transformation

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ABSTRACT

We have identified that StarD10, a member of the START protein family, is overexpressed in both mouse and human breast tumors. StarD10 was initially discovered on the basis of its cross-reactivity with a phosphoserine-specific antibody in mammary tumors from Neu/ErbB2 transgenic mice and subsequently isolated from SKBR3 human breast carcinoma cells using a multistep biochemical purification strategy. We have shown that StarD10 is capable of binding lipids. StarD10 was found to be overexpressed in 35% of primary breast carcinomas and 64% of human breast cancer cell lines, correlating with their ErbB2/ErbB2 status. Coexpression of StarD10 with ErbB2/epidermal growth factor receptor in murine fibroblasts enhanced anchorage-independent growth in soft agar, providing evidence for functional cooperation between StarD10 and ErbB receptor signaling. Taken together, these data suggest that overexpression of this lipid-binding protein contributes to breast oncogenesis.

INTRODUCTION

The ErbB family of receptor tyrosine kinases plays a critical role in the pathogenesis of breast cancer. Amplification and/or overexpression of HER2/ErbB2 occurs in 20–30% of human breast cancers and correlates with poor prognosis (1). Deregulated expression of HER1/ ErbB1/epidermal growth factor (EGF) receptor has also been observed in breast cancer, often in association with aberrant expression of ErbB2 (2, 3). ErbB1 and ErbB2, together with HER3/ErbB3 and HER4/ErbB4, constitute the ErbB family of receptor tyrosine kinases. These receptors signal cooperatively by forming ligand-induced combinations of homo- and hetero-dimers (4, 5). The complex network of ErbB receptor-ligand combinations provides precise signaling along pathways underlying diverse developmental processes (6–8).

The importance of ErbB2 signaling in mammary tumorigenesis has been established using transgenic mouse models. Overexpression of neu, the rat homologue of ErbB2, from the mouse mammary tumor virus promoter induces focal mammary tumors which frequently metastasize to the lung (9). The long tumor latency period in these mice suggested that additional molecular events were required for mammary tumors to develop. Further analysis of these tumors identified somatic mutations in neu, resulting in constitutive activation of the receptor (10). Up-regulation of ErbB3 protein in these tumors suggested that ErbB2 synergizes with ErbB3 in oncogenesis (11). In human breast carcinoma cells that overexpress ErbB2, down-regulation of ErbB3 using an artificial erbB3-specific transcriptional repression revealed that ErbB3 is essential for ErbB2-mediated proliferation (12). The ErbB2/3 heterodimer appears to have potent mitogenic and transforming properties in vitro (13, 14) and leads to efficient activation of the phosphatidylinositol 3-kinase (PI3K) pathway (15, 16). Signaling through PI3K plays an important role in cellular survival by phosphorylating and inactivating growth-inhibitory and proapoptotic proteins, including the FOXO transcription factors (17). In addition, the ErbB2 and ErbB3 receptors recruit the adaptor proteins Shc and Grb2 (16, 18, 19), resulting in stimulation of the Ras-Raf-mitogen-activated protein kinase pathway (20, 21), which has been implicated in mammary tumor progression (22, 23). Furthermore, Src kinase activity is enhanced in both mammary tumors from Neu transgenic mice (24) and human breast tumors (25, 26), and Src cooperates with ErbB1 in the transformation of mouse fibroblasts (27). Thus, aberrant expression of ErbB receptors triggers the activation of multiple downstream effectors, in addition to synergizing with other proto-oncogenes to transform mammary epithelium.

The Neu mouse model has served as a valuable model for the identification of intracellular mediators of ErbB2-induced tumor development. Although ErbB receptor signaling has been studied extensively, little is known about the deregulation of transcription factors in ErbB2-induced tumorigenesis. In the course of analyzing the expression and activation of the forkhead transcription factor FKHR/FOXO1 in Neu transgenic mice, we observed a novel protein band that was specifically recognized by a phospho-FKHR antibody. Interestingly, this M 35,000 protein was abundant in tumors derived from Neu transgenic mice but not adjacent normal tissue. Here, we describe the isolation and characterization of this phosphoprotein, StarD10, a member of the START domain family of lipid-binding proteins. StarD10 was found to be coexpressed with ErbB2 in many breast carcinoma lines and cooperated with the ErbB pathway in cellular transformation. Finally, StarD10 overexpression was observed in a high proportion of primary breast cancers, supporting a role for this lipid-binding protein in deregulated cell growth and tumorigenesis.

MATERIALS AND METHODS

Reagents. Antibodies used were polyclonal antiphospho-FKHR (S256) antibody (New England Biolabs, Beverly, MA), mouse anti-α-tubulin monoclonal antibody (clone B-5-1-2; Sigma, St. Louis, MO), mouse anti-ErbB2 monoclonal antibody (Ab-10; Neomarkers, Fremont, CA), rabbit anti-ErbB2 polyclonal antibody (A0485; DAKO, Carpinteria, CA), mouse anti-Neu monoclonal antibody (Ab-3; Calbiochem, Darmstadt, Germany), mouse anti-Flag monoclonal antibody (clone B-5-1-2; Sigma, St. Louis, MO), mouse anti-ErbB2 monoclonal antibody (New England Biolabs). Antibodies used were polyclonal antiphospho-FKHR (S256) antibody (New England Biolabs, Beverly, MA), mouse anti-α-tubulin monoclonal antibody (clone B-5-1-2; Sigma, St. Louis, MO), mouse anti-ErbB2 monoclonal antibody (Ab-10; Neomarkers, Fremont, CA), rabbit anti-ErbB2 polyclonal antibody (A0485; DAKO, Carpinteria, CA), mouse anti-Neu monoclonal antibody (Ab-3; Calbiochem, Darmstadt, Germany), mouse anti-Flag monoclonal antibody (Sigma), and rabbit anti-Pet-p1 monoclonal antiserum (kindly provided by Y. Nishimoto, Osaka University, Osaka, Japan).

Cell Culture and Mouse Strains. The majority of breast epithelial cell lines was maintained in RPMI containing 10% fetal bovine serum (Commonwealth Serum Laboratory). MCF10A cells were grown in DMEM/F12, supplemented with 10% fetal bovine serum, 10 ng/ml EGF (Sigma), 5 μg/ml insulin (Sigma), and 1 μM dexamethasone (Sigma). HER14 (NH3T3 cells transfected with human ErbB1) was a gift from R. Daly (Garvan Institute, Sydney, Australia). HER14, 293T, and Bosc cell lines were grown in DMEM containing 10% fetal bovine serum. HER14 StarD10 transductants were generated by infection with a pBabe retrovirus encoding hemagglutinin (HA)-tagged StarD10 into Bosc packaging cells. Virus was collected, then used to infect HER14 NH3T3 cells, and selected with 1 μg/ml puromycin (Sigma), thus generating HER14-StarD10 transductants. For transient transfections,
293T cells were transfected with Fugene reagent (Roche, Penzberg, Germany) according to the manufacturer’s instructions. Mouse mammary tumor virus-negative mice have been described previously (9). Nulliparous female mice were aged for 8–12 months in standard animal facilities at The Walter and Eliza Hall Institute and sacrificed when tumors developed.

Cloning of StarD10. Total RNA was extracted from SKBR3 cells using RNAzol (Tel-Test, Friendswood, TX). First-strand cDNA synthesis was performed, and StarD10 cDNA was amplified by PCR using primers based on the CGL-52 sequence (accession no. AF158110; Ref. 28). StarD10 cDNA was cloned into Flag-pEF-PGKpuro and pBabePuro vectors for expression in mammalian cells.

Protein Extraction of Cells and Tissues. For cytosolic protein extraction, cells were lysed in hypotonic buffer [10 mM HEPES (pH 7.9), 130 mM sorbitol, 0.5 mM sodium fluoride, and 0.5 mM β-glycerophosphate plus Complete protease inhibitors; Roche], left to swell on ice for 10 min, homogenized by douncing, and then centrifuged at 800 × g for 10 min. The pellet was washed with hypotonic buffer and the supernatants were combined to yield the cytosolic fraction. Whole-cell extracts were obtained by solubilizing cells in NP40 extraction buffer (NEB) [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 20 mM β-glycerophosphate plus Complete protease inhibitors]. Lysates were clarified by centrifugation at 16,000 × g for 10 min. Protein lysates of mammary tissue were obtained by grinding the tissue to a fine powder under liquid nitrogen and subsequent solubilization in Triton X-100 extraction buffer (TEB) (see above, substitution of NP40 with Triton X-100). Lysates were homogenized by passing through a 21-gauge needle before clarification by centrifugation. Lysates from organs were prepared by resuspending cells in 20 mM Tris (pH 7.4), 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, and 10% glycerol with Complete protease inhibitors and processed as described for whole-cell extracts.

Immunoprecipitation and Western Blotting. For immunoprecipitations, equal amounts of protein were incubated with specific antibodies for 2 h on ice. Immune complexes were collected with protein G-Sepharose (Amersham Pharmacia, Buckinghamshire, United Kingdom) and washed three times with NEB (see above). For phosphorylation analysis, immune complexes were further washed with 10 mM Tris (pH 8) and 100 mM NaCl before incubation in the same buffer with five units of calf intestinal phosphatase (Roche) for 45 min at 37°C. Precipitated proteins were released by boiling in sample buffer and subjected to SDS-PAGE using 4–20% gradient gels (Novex, Carlsbad, CA). The proteins were blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with 20% horse serum (Hunyer, Jesmond, NSW, Australia) in PBS containing 0.1% Tween 20, filters were probed with specific antibodies. Proteins were visualized with peroxidase-coupled secondary antibody using the enhanced chemiluminescence detection system (Amersham). Stripping of membranes was performed in SDS buffer [62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol] for 30 min at 60°C. Membranes were then reprobed with the indicated antibodies.

Free Flow Electrophoresis (FFE). FFE was essentially performed as described in Hoffmann et al. (29) using the Octopus apparatus (Dr. Weber, Kirschheim GmbH, Germany). The isoelectric focusing running buffer was aqueous 0.2% w/v hydroxypropyl methyl cellulose and 0.2% w/v carrier ampholytes (Servalyte pH 3–10). Electrode solutions were 100 mM H₃PO₄ (anode) and 50 mM NaOH (cathode); the counter flow solution (0.7 liter/min) was 0.2% hydroxypropyl methyl cellulose containing 0.02 M t-arginine and 0.02 M t-lysine. The sample was diluted to a concentration of ~0.25 mg of protein/ml, and electrophoresis was performed at 4°C with a flow rate of 1.4 ml/min and constant voltage of 1250 V (9). A linear gradient from 0 to 100% B was applied at a flow rate of 100 μl/min, and 100–500 μl fractions were collected (A, 0.1% trifluoroacetic acid; B, 60% n-propyl alcohol). Preparative RP-HPLC was performed on a 1090 (Hewlett Packard, Palo Alto, CA) using a Brownlee RP 300 (100 × 4.6 mm, 300 Å, 15 μm). A linear gradient from 0 to 100% B was applied at a flow rate of 100 μl/min, and 100–500 μl fractions were collected (A, 0.1% trifluoroacetic acid; B, 60% n-propyl alcohol).

Protein Precipitation and Preparative SDS-PAGE. HPLC fractions containing the M, 35,000 protein were pooled and precipitated with 0.5% deoxycholate and 15% trichloroacetic acid (TCA) plus two volumes of acetone at −20°C. After centrifugation at 16,000 × g for 15 min, the protein pellet was washed with acetone, dissolved in sample buffer, and loaded onto a large 10% SDS-PAGE gel. The gel was fixed and stained with Coomassie Phast-gel Blue R (Amersham Pharmacia).

Im-Gel Tryptic Digest and Mass Spectrometry. Coomassie-stained bands were destained with 50 mM NH₄HCO₃/50% acetonitrile (1:1) and digested with modified porcine trypsin (Promega, Madison, WI). The sample was then concentrated on a Zip Tip μ-C18 (Millipore) and eluted into nanospray capillaries (MDS Proteomics, Charlottesville, VA) with 20–60% methanol containing 5% formic acid. Mass spectrometry was performed on a Pulsar ion quadrupole Time of Flight (TOF) mass spectrometer (Applied Biosystems, Foster City, CA) with a nanoelectrospray ion source (MDS Proteomics). Product ion scans were acquired using collision energies that retained 10% of the intensity of the precursor ion. Arsen was used as collision gas at a recorded pressure of 4.3 × 10⁻⁵ Torr. Tandem mass spectroscopy data were searched via the Mascot search engine (30) or sequenced de novo.

StarD10-Lipid Interaction by Intrinsic Fluorescence Measurement. StarD10 cDNA was cloned into pGEX-6p (Amersham Pharmacia) and transformed into BL21 bacteria to produce a glutathione S-transferase StarD10 fusion protein. Glutathione S-transferase-StarD10 expression was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside for 4 h at 30°C. Bacteria were harvested and resuspended in PBS containing 50 μg/ml lysozyme, Complete protease inhibitors, 10 mM sodium fluoride, and 20 mM β-glycerophosphate. Triton was added to a final concentration of 1% before two freeze-thaw cycles and sonication. Glutathione S-transferase-StarD10 was purified from the clarified lysate with glutathione resin and cleaved with Prescission protease according to the manufacturer’s instructions (Amersham Pharmacia). The purity of cleaved StarD10 protein was verified by SDS-PAGE and Coomassie staining. Fluorescence measurements were on an Amino Bowman spectrometer series 2 (Rochester, NY). Trypophan and tyrosine were excited at 280 nm, and emission spectra were recorded from 290 to 400 nm (band widths: 4 nm). Porcine brain lipids (Avanti Polar Lipids, Alabaster) were dried under nitrogen, and small unilamellar vesicles were prepared by sonication to yield a final lipid concentration of 1 mM in 20 mM HEPES (pH 7.2), 100 mM NaCl, and 5 mM EDTA.

Northern Blotting. Poly(A)⁺ RNA was isolated from breast epithelial cell lines, and Northern analysis was performed as described (31). Briefly, 3 μg of poly(A)⁺ RNA were fractionated on 1% agarose-formaldehyde gel, transferred to Hybond N⁺ (Amersham Pharmacia Biotech), and hybridized with the following cDNA probes: (a) StarD10 cDNA, encompassing the entire 876-bp coding region; (b) ErbB2 cDNA (residues 1–228); or (c) glyceraldehyde-3-phosphate dehydrogenase cDNA. A human multiple tissue poly(A)⁺ RNA Northern blot (Clontech Laboratories, Inc.) was hybridized with a StarD10 cDNA probe.

Generation of StarD10-Specific Polyclonal Antibodies. Rabbits were immunized with StarD10 (264–277)-C²⁶- peptide ESAVAESSREEMG (Astrup, Parkville, Victoria, Australia) coupled to keyhole limpet hemocyanin (Sigma). Antibody was affinity purified with peptide that had been covalently coupled to Sulfolink-coupling gel (Pierce, Rockford, IL). Elution was with 100 mM glycine buffer (pH 2.7), and neutralized antibody-containing fractions were pooled and dialyzed against PBS.

Immunohistochemistry. Tissue arrays (Clinomics, Pittsfield, MA) were blocked in PBS containing 5% goat serum (Hunter) and 0.05% Tween 20 and then incubated with affinity-purified anti-StarD10 antibody, followed by incubation with biotinylated antirabbit IgG and horseradish peroxidase-Streptavidin (DAKO), before detection with diaminobenzidine (DAKO). Staining and scoring with anti-ErbB2 antibody (DAKO) were performed using standard protocols (32). All antibody incubations were carried out in PBS containing 5% goat serum and 0.05% Tween 20. Slides were counterstained with hematoxylin.

Soft Agar Assays. HER14 cells (2 × 10⁵) were plated in triplicate (six-well plates) in 2 ml of culture medium, supplemented with 0.5% DIFCO agar overlaying a 0.7% agar cushion. Cells were stained by the addition of 400 μl of PBS containing 0.5 mg of nitroblue tetrazolium (Sigma) for 24 h before photographing using a Nikon SMZ-U microscope connected to an Axioacam digital camera (Zeiss, Jena, Switzerland). Photographs were analyzed using ImageJ software (National Institutes of Health).
RESULTS

A Phosphospecific FKHR Antibody Cross-Reacts with a Tumor-Specific M, 35,000 Protein. The FOXO subgroup of forkhead transcription factors consisting of FKHR, FKHRL1, and AFX were recently identified to be regulated by the PI3K pathway (17, 33). These proteins drive the expression of cell cycle regulators, such as p27, and apoptotic proteins, including Bim and Fas ligand (17, 33). Phosphorylation on specific serine and threonine residues by protein kinase B/Akt leads to their inactivation and sequestration in the cytoplasm by 14-3-3-scaffolding proteins, thereby preventing their negative effects on cell proliferation.

To explore the potential inactivation of the FKHR transcription factor (FOXO1) by ErbB receptor signaling, mammary tumor lysates derived from mouse mammary tumor virus-neu transgenic mice were analyzed by Western blotting using an antiphosphoprotein antibody to FKHR phosphorylated on serine 256 (Fig. 1A). No signal corresponding to FKHR was detected in tumor or adjacent mammary tissue. Interestingly, the antibody strongly cross-reacted with a M, 35,000 protein that was present in tumor but not detectable in adjacent or normal mammary tissue (Fig. 1A). Immunoblotting with an anti-Neu/ErbB2 antibody confirmed overexpression of the transcripts in these tumors. Further analysis of several breast epithelial cell lines revealed high levels of the M, 35,000 protein in the majority (6 of 11) of transformed epithelial lines (see Fig. 4B). Because no FOXO family members corresponding to this molecular weight had been described, we speculated that the M, 35,000 protein might represent a novel FKHR-related protein. This protein was likely to be phosphorylated on a serine residue lying in a conserved sequence context, given the specificity of the FKHR-specific antibody.

Purification of the M, 35,000 Protein. Because the phospho-FKHR antibody failed to immunoprecipitate the M, 35,000 protein, we devised a multistep biochemical purification strategy to purify the protein from human breast carcinoma SKBR3 cell lysates, which contain high levels of this protein. This strategy combined FFE, a continuous liquid-based, isoelectric-focusing method (29), RP-HPLC, and SDS-PAGE. By FFE, we identified two protein bands with isoelectric-focusing points of pH 5.6 and pH 6.0 (Fig. 1B), which may represent differential phosphorylation states of the protein. The M, 35,000 protein was also shown to elute in specific RP-HPLC fractions (Fig. 1C). After preparative FFE purification of the M, 35,000 protein from SKBR3 cytosolic protein, the pooled fractions containing the M, 35,000 protein were subjected to RP-HPLC and then SDS-PAGE (Fig. 1D). Bands migrating at M, 35,000 were excised, digested with trypsin in situ, and analyzed by tandem mass spectrometry. All 10 peptides identified from the indicated band in Fig. 1D were found to match Q9Y365 (TrEMBL database; Fig. 2B), which corresponds to a human cDNA sequence conserved between the Caenorhabditis elegans proteome and human expressed sequence tag nucleotide databases (28). Q9Y365 is the human homologue of mouse Pctp-1 (phosphatidylcholine transfer protein-like), which was cloned from a testis library (34) and has been assigned the formal name StarD10. The predicted amino acid (aa) sequence did not contain a forkhead domain, but a putative serine phosphorylation site resembling S256 in FKHR was identified (Fig. 2A). This provides a possible explanation for the observed cross-reactivity with the phospho-FKHR antibody.

The M, 35,000 Protein Shares Homology with Lipid-Binding Proteins. Sequence analysis of Q9Y365 predicted a molecular weight of M, 40,000. Given that no sequence coverage of the first predicted 71 aa was obtained, it seemed likely that translation initiated from methionine 69, giving rise to a protein of 291 residues with a theoretical mass of M, 33,000 (isoelectric point of 6.7; Fig. 2B). This shorter version of Q9Y365 has recently been deposited into the Swiss-Prot database. To test whether this protein cross-reacted with the phospho-FKHR antibody, we cloned the corresponding cDNA (from SKBR3 cells) into the pElFpPGKuro expression vector harboring an NH2-terminal Flag epitope tag. Indeed, transient transfection of this construct into 293T cells gave rise to a protein that was recognized by the phospho-FKHR antibody (Fig. 2C). The epitope tag accounted for the slight difference in size compared with the endogenous protein in SKBR3 cells. Recognition of this protein proved to be phosphorylation dependent, because treatment of immunoprecipitated Flag-tagged StarD10 with alkaline phosphatase abolished cross-reactivity (Fig. 2D). This finding further indicates that serine 259 in StarD10 is likely to be phosphorylated.

Database searches identified the presence of a START domain (35, 36) in StarD10 (aa 21–226), followed by a COOH-terminal tail of 65 aa. The START domain refers to a lipid-binding region first described in the steroidogenic acute regulatory protein Star (37, 38). Although 15 START domain proteins have been identified in mammals thus far, lipid binding has only been established for a few members. To test the functionality of the START domain within StarD10, we made use of the intrinsic fluorescence of the protein. StarD10 contains multiple tyrosine and tryptophan residues that can be excited at 280 nm. A change in the fluorescence emission spectrum in the presence of a binding partner is indicative of conformational changes within the protein. In the absence of lipids, recombinant StarD10 protein purified from bacteria displayed a maxima fluorescence emission at 335 nm (Fig. 2E). On addition of small unilamellar vesicles prepared with lipids extracted from porcine brain, a decrease in the intensity of fluorescence was observed (Fig. 2F). This finding suggests that a conformational change has occurred in StarD10 upon interaction of the protein with membranes, indicating that StarD10 is capable of binding lipids.
Expression Analysis of StarD10. Northern analysis of StarD10 mRNA from a variety of human organs revealed high levels of a 1.4-kb transcript in liver, heart, skeletal muscle, and kidney, as also observed for the mouse homologue (Ref. 34; Fig. 3A). Although a faint signal was detected in placenta, organs, including the brain, colon, thymus, spleen, small intestine, lung, and peripheral blood lymphocytes, lacked StarD10 mRNA. Examination of StarD10 expression in mouse tissues by immunoblotting confirmed high expression in liver. Lower levels of StarD10 were detected in kidney, salivary gland, testis, and colon (Fig. 3B). In the mammary gland, expression of StarD10 was found to be differentially regulated at different developmental stages. StarD10 was most abundant during pregnancy and lactation, with low expression evident in the virgin state (Fig. 3C).

Coexpression of StarD10 and ErbB2 in Breast Carcinoma Lines. To generate an antibody specific for human StarD10, we immunized rabbits with a peptide encompassing aa 265–277 at the COOH terminus of this protein. Affinity-purified antibody specifically recognized the human StarD10 protein. Prominent expression of StarD10 was originally observed in tumors from mouse mammary tumor virus-neu transgenic mice and human breast carcinoma cell lines using a phospho-FKHR-specific antibody. To determine whether StarD10 was post-translationally modified in these carcinoma cell lines, we analyzed StarD10 by Western blotting using both phospho-FKHR- and StarD10-specific antisera (Fig. 4A). All breast carcinoma cell lines that cross-reacted with the phospho-FKHR antibody were found to express high levels of StarD10, indicating that the protein is overexpressed, and serine 295 is constitutively phosphorylated. Intriguingly, most cell lines that expressed ErbB2 also displayed high levels of StarD10 protein.

We next sought to determine whether StarD10 up-regulation occurred at the RNA level and performed Northern analysis on a panel of breast epithelial cell lines (Fig. 4B). High levels of StarD10 mRNA were found in the cell lines that express StarD10 protein, indicating that increased expression occurs at the transcriptional level. Consistent with the results obtained by Western blotting, StarD10 mRNA was almost negligible in both human (184, HBL100) and mouse (SCp2, Eph4, HC11) immortalized cell lines. Strikingly, all cell lines that overexpressed ErbB2 mRNA were found to have high StarD10 levels.

StarD10 expression, however, was also detected in cell lines that did not overexpress ErbB2.

StarD10 Is Overexpressed in a Subset of Primary Human Breast Cancers. To examine whether StarD10 was up-regulated in primary breast cancers, we performed immunohistochemistry on tissue arrays comprising 79 invasive breast cancers. Tumor specimens were scored as either low/negative or moderate/high for expression of StarD10, based on the intensity of staining with an affinity-purified StarD10 antibody. A high proportion of tumors (28 of 79; 35%)
yielded moderate to intense staining and included infiltrating ductal, lobular, and mixed carcinomas. Four infiltrating ductal breast carcinomas exhibiting intense staining of the nucleus and cytoplasm are shown in Fig. 5, C–F. In contrast, negligible StarD10 expression was observed in normal breast tissue (Fig. 5, A and B). Immunostaining with an anti-ErbB2 antibody revealed that of the eight tumors displaying strong membranous staining, four also expressed StarD10 at high levels. Two examples are depicted in Fig. 5, in which the StarD10-expressing tumors E and F correspond to the ErbB2-positive tumors G and H, respectively.

**StarD10 Cooperates with ErbB1 in Agar Assays.** To investigate the effect of overexpression of StarD10 on cellular transformation, we generated NIH3T3 and HER14 cells stably expressing StarD10 by retroviral infection. The HER14 line corresponds to NIH3T3 cells that express ErbB1; these and parental NIH3T3 cells lack detectable StarD10 by Western blotting. Transduction of HER14 cells with StarD10 resulted in expression similar to that seen for StarD10 in breast carcinoma cell lines (Fig. 6A). Overexpression of StarD10 did not change the morphology of NIH3T3 or HER14 cells, nor was growth altered in either normal and limiting serum conditions (data not shown). Although StarD10 alone was not sufficient to cause transformation of NIH3T3 cells, it was found to enhance anchorage-independent growth of HER14 cells in soft agar (Fig. 6B). Quantification of digital photographs using image analysis software revealed that StarD10 expression increased both the number and size of colonies (Fig. 6B). This was confirmed in multiple experiments and using independent pools of StarD10 HER14 transductants. These results demonstrate that StarD10 cooperates with ErbB signaling in eliciting anchorage-independent growth.

**DISCUSSION**

Aberrant expression of the ErbB2 receptor tyrosine kinase has been implicated in the pathogenesis of a high proportion of sporadic breast cancers (2, 3). Moreover, targeted expression of ErbB2/Neu to the mammary gland leads to tumor development in mice (9). Here, we report the isolation of the phosphoprotein StarD10 and its up-regulation in mouse mammary tumors, human breast carcinoma cell lines (9 of 14), and primary breast cancers (35%). A striking correlation between ErbB2 status and StarD10 expression was found in Neu tumors and human breast cancer cell lines. Moreover, StarD10 was demonstrated to cooperate with ErbB1 in cellular transformation.
assays, in which an increase in both the number and size of colonies was evident. In primary carcinomas, 50% (4 of 8) of ErbB2-positive tumors expressed high levels of StarD10. These data indicate a strong correlation between StarD10 and ErbB2 overexpression, suggesting a selective growth advantage for tumors expressing both proteins. Despite coexpression of StarD10 and ErbB2 in breast cancer cell lines and a subset of breast tumors, StarD10 does not appear to be a direct transcriptional target of the receptor. In NIH3T3 cells that express constitutively active Neu (NeuT), no increase in the level of StarD10 protein was observed (data not shown). The molecular basis of the apparent coexpression of StarD10 and ErbB2 remains to be established but may involve regulation by a common transcription factor. Interestingly, StarD10 localizes to human chromosome 11q13, in proximity to the amplicon comprising the cyclin D1 and EMS genes, which are frequently overexpressed in breast cancer (39). However, the mechanism underlying StarD10 overexpression in breast cancer appears to reflect increased transcription rather than gene amplification, because Southern analysis of DNA from normal and human breast cancer cell lines revealed no alterations (data not shown).

Overexpression of StarD10 may occur in additional tumor types, such as colon carcinoma. Screening of a cDNA expression library derived from a human colon tumor patient with autologous serum identified autoantibodies against an expressed sequence tag corresponding to StarD10 (40). This finding indicates that StarD10 may be a target for antitumoral immune response. Furthermore, we have evidence that StarD10 is up-regulated in a significant proportion of human colon carcinoma cell lines (data not shown), indicating a potential role in the development of this cancer. ErbB1 is frequently overexpressed in colorectal cancer. However, there is no apparent correlation between StarD10 expression and that of either ErbB1 or ErbB2 in these cell lines. StarD10 may therefore synergize with other oncogenes in colon cell neoplasia.

On the basis of our data using the phospho-FKHR-specific antibody, StarD10 appears to be constitutively phosphorylated on serine 259 in proliferating breast epithelial cells. This antibody recognizes FKHR phosphorylated on serine 256, a site targeted by PKB/Akt. However, the Ser259 site in StarD10 does not have the appropriate arginine determinants for protein kinase B/Akt, and treatment with the PI3K inhibitor LY294002 had no effect on phospho-FKHR antibody recognition of StarD10. The kinase responsible for phosphorylation of StarD10 at this site is not yet known. Functionally, the StarD10 S259A mutation has little effect on promoting anchorage-independent growth but may be important for regulating protein turnover, because the mutant protein was expressed at a lower level (data not shown).

Of the 15 mammalian START domain-containing proteins, which have been assigned the formal names StarD1–15, Pctp-I/StarD10 displays the highest homology with the Pctp subfamily, consisting of Pctp/StarD2, GTT1/StarD7, and GPBP/StarD11 (41). The START domain protein MLN64 is coamplified with ErbB2 in human tumors (42). Similar to STAR, the founding member of this family, MLN64 is a cholesterol transfer protein that is thought to deliver cholesterol to the inner mitochondrial membrane, where it serves as a metabolic precursor for steroid hormones (43–45). The up-regulation of MLN64 has therefore been proposed to contribute to intratumoral steroidogenesis. Pctp exclusively binds PC (46). The quenching of the intrinsic fluorescence of StarD10 by brain lipid liposomes (Fig. 2E) indicates that StarD10 also interacts with lipids. Preliminary data suggest that StarD10 can bind phosphatidylcholine (6), but its specificity for other lipids remains to be defined.

Both StarD10 and Pctp are particularly abundant in the liver, where they may be involved in export of lipids into bile (47, 48). The presence of high levels of StarD10 in the lactating mammary gland suggests a potential physiological role in the functional differentiation of this organ. Within the cell, lipids are used by phospholipases to generate second messengers, such as phosphatidic acid and diacylglycerol (49). StarD10 may play a role in replenishing membranes with specific lipids metabolized by phospholipases, and deregulation of this transport may lead to aberrant lipid signaling, thereby contributing to cellular transformation. The phosphatidylinositol transfer protein (50) has been demonstrated to influence EGF signaling, whereby EGF-induced phosphorylation of phosphatidylinositol 3-phosphate and subsequent hydrolysis by phospholipase C γ was shown to require the presence of phosphatidylinositol transfer protein (50). StarD10 fulfills a similar function as a cofactor for receptor-mediated lipid signaling. In HER14 cells, the expression of StarD10 did not increase EGF-induced PI3K activation (data not shown), but it may affect other signaling pathways. Alternatively, the ability of StarD10 to stimulate anchorage-independent growth and cooperate with ErbB receptors may be independent of its lipid-binding function. StarD10 has a 65 aa COOH-terminal extension that is not present in Pctp and may confer an additional function. Elucidation of the functional domains within StarD10 will provide insight into the mechanism by which this protein acts.

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