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Elevated content of the tyrosine kinase substrate phospholipase C-γ1 in primary human breast carcinomas

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ABSTRACT **Phospholipase C-γ1 (PLC-γ1) is a substrate for several receptor tyrosine kinases and its catalytic activity is increased by tyrosine phosphorylation. However, the biological significance of this molecule in normal or malignant human epithelial cell proliferation is unknown. We determined the relative content of PLC-γ1 in primary human mammary carcinomas and in nonmalignant mammary tissues. By Western blot and immunohistochemistry, considerably higher levels of PLC-γ1 protein were detectable in the majority of carcinomas and in one of two benign fibroadenomas compared to normal breast tissues. In 18 of 21 carcinomas that contained high levels of PLC-γ1, the presence of phosphotyrosine on PLC-γ1 could also be detected. All carcinomas in which tyrosine phosphorylated PLC-γ1 was present also expressed detectable levels of the epidermal growth factor receptor or erbB-2, two tyrosine kinases known to phosphorylate this enzyme. Thus, a high percentage of mammary carcinomas concomitantly display increased levels of receptor tyrosine kinases and a direct tyrosine phosphorylation substrate, thereby potentially amplifying two successive steps in a signal transduction pathway.**

Recently, several reports have demonstrated that phospholipase C-γ1 (PLC-γ1) is a direct substrate of the epidermal growth factor (EGF) receptor protein tyrosine kinase (1–4). PLC isoenzymes hydrolyze phosphatidylinositol 4,5-bisphosphate to produce two intracellular second messengers: inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (5), which regulate intracellular levels of Ca^{2+} and protein kinase C activity, respectively. As much as 50–70% of the total cellular pool of PLC-γ1 becomes tyrosine phosphorylated within 1 min after the addition of EGF to cells (2, 3, 6). Importantly, growth factor-induced tyrosine phosphorylation increases the catalytic activity of PLC-γ1 (7, 8). *In vitro* experiments show that the purified EGF receptor phosphorylates PLC-γ1 but does not significantly phosphorylate the PLC-β and δ isoenzymes (4). However, not all tyrosine kinases phosphorylate PLC-γ1. Although the platelet-derived growth factor (PDGF) (3, 9), fibroblast growth factor (10), nerve growth factor (11), and erbB-2 receptors (12, 13) also phosphorylate PLC-γ1, insulin and colony-stimulating factor type 1 receptors do not utilize PLC-γ1 as a substrate (3, 14, 15). Taken together these data suggest that phosphorylation of PLC-γ1 is a biologically important event in growth factor-stimulated inositol phospholipid metabolism and perhaps plays a role in signal transduction for cell proliferation. However, most of these studies have utilized cultured A-431 human squamous carcinoma cells or mouse 3T3 fibroblasts transfected with wild-type human EGF receptors. Studies in human tissues are lacking and, although PLC-γ1 is ubiquitously distributed in animal tissues, factors that may influence its level of expression are unknown.

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To provide evidence for a functional role of PLC-γ1 in proliferating human epithelial tissues, we have examined PLC-γ1 levels in benign and malignant mammary epithelium. The EGF and erbB-2 receptors, both of which tyrosine phosphorylate PLC-γ1, are overexpressed in some mammary carcinomas and are associated with a more aggressive cancer behavior and a poor patient prognosis (16–20). If PLC-γ1 was functionally important for the biological effects of these receptor tyrosine kinases, one could speculate on higher levels of this isozyme in those tissues with EGF receptor- or erbB-2-dependent cell proliferation.

MATERIALS AND METHODS

Collection and Processing of Surgical Specimens. After surgical resection, samples were quick-frozen in liquid nitrogen and stored at −80°C. Thirty invasive breast adenocarcinomas and 17 nonmalignant mammary tissues were available for this study. The nonmalignant tissues included 6 reduction mammoplasty specimens, 2 benign fibroadenomas, 1 fibrocystic disease specimen, and 8 tumor-free samples adjacent to a resected carcinoma. Tissues were ground in a Polytron homogenizer (Brinkmann) on ice in a hypotonic buffer [20 mM Hepes, pH 7.4/5 mM EGTA/1 mM $MgCl_2$/1 mM sodium orthovanadate/1 mM phenylmethylsulfonyl sulfate (PMSF)/1 μg each of aprotinin, pepstatin, and leupeptin per ml] and centrifuged at 1000 × *g* for 10 min at 4°C; the pellet was discarded. A membrane fraction was obtained by centrifuging the supernatant at 100,000 × *g* for 45 min at 4°C. The particulate membrane fraction was solubilized in a buffer containing 1% Triton X-100, 50 mM Hepes (pH 7.5), 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μg each of aprotinin, pepstatin, and leupeptin per ml. The supernatant cytosolic fractions from the ultracentrifugation were concentrated by lyophylization, reconstituted in the same buffer as the membranes, and stored at −80°C until further use.

Preparation of PLC-γ1 Antiserum. To generate a PLC-γ1 antibody, 5 mg of a synthetic peptide SFEARYQQPFEDFR(C) from bovine PLC-γ1 (residues 1249–1262), containing an added cysteine at the carboxyl terminus, was coupled to 4 mg of keyhole limpet hemocyanin using maleimidobenzoyl-*N*-hydroxysuccinimide as described (21). This sequence does not appear in PLC-γ2 (22) and is retained in human PLC-γ1 (10) with one change—i.e., A to S, at residue 1252. Rabbits were immunized and given a booster injection; either crude serum or the IgG fraction, purified by DEAE-Affi-Gel blue chromatography (Bio-Rad), was used. Confirming its specificity, the antiserum did not precipitate PLC from HL-60 cell lysates. These cells contained PLC-γ2 but not PLC-γ1.

Abbreviations: PLC, phospholipase C; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

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PLC-γ1 Immunoblot and Immunohistochemistry. For Western blots, membrane and cytosolic fractions from mammary tissues were subjected to 7% SDS/PAGE and transferred to nitrocellulose. Membranes were then incubated with a 1:500 dilution of the PLC-γ1 antiserum; this was followed by incubation with ^{125}I-labeled donkey anti-rabbit IgG (≈200,000 cpm/ml; Amersham). The immunodetected PLC-γ1 bands were visualized by autoradiography.

For immunohistochemistry, 5- to 6-μm frozen sections of each tumor or normal tissue were cut in a cryostat at −20°C, thaw-mounted onto 3-triethoxysilylpropylamine-coated microscope slides, and then fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS). Sections were rinsed and used immediately or stored in 80% ethanol at −20°C overnight. After dehydration in graded alcohols, endogenous peroxidase activity was quenched with absolute methanol containing 3% H_2O_2 for 30 min. Sections were rehydrated with PBS and subsequently incubated for 10 min in 10% normal goat serum in PBS containing 0.1% bovine serum albumin. After blotting off the excess blocking serum, the polyclonal PLC-γ1 antiserum (1:50 dilution) was added for 2 hr at room temperature. In each case a similar dilution of PLC-γ1 antiserum that had been preadsorbed with immunizing peptide (1 mg/ml) was used as a negative control. Frozen sections of MDA-468 human breast cancer cell xenografts and MDA-468 cells plated on sterile microscope slides were used as positive controls. PLC-γ1 tissue immunoreactivity was detected using a biotinylated swine anti-rabbit antiserum (DAKO, Carpinteria, CA; 1:200 dilution) for 1 hr; this was followed by incubation with peroxidase-conjugated avidin-biotin complex (DAKO) for 30 min. After rinsing twice in PBS, the sections were incubated with 3-aminoethylcarbazole (Biomedia, Foster City, CA) for 10 min and then counterstained in hematoxylin and 1.2% Li_2CO_3.

Phosphotyrosine Immunoprecipitation. A known amount of cytosolic protein from benign and malignant tissue extracts, in a buffer containing phosphatase and protease inhibitors (see above), was adsorbed onto 200 μl of a Sepharose-linked anti-phosphotyrosine (monoclonal 1G2) bead matrix (23) for 2–4 hr at 4°C with rocking as described (24). After washing, the specifically adsorbed phosphotyrosyl proteins were eluted with 20 mM phenylphosphate, electrophoresed, and subjected to PLC-γ1 immunoblot. To assess the specificity of the anti-phosphotyrosine matrix, the tissue cytosols were immunoprecipitated with the anti-phosphotyrosine matrix in the presence of excess phenylphosphate or phosphotyrosine prior to elution and PLC-γ1 immunoblot.

EGF Receptor and erbB-2 Immunodetection. Membrane fractions from the mammary tissue extracts were subjected to a Western blot procedure for EGF receptors or erbB-2. For EGF receptors, we utilized a 1:1000 dilution of rabbit antiserum 310 (25) and for erbB-2 immunodetection we used a 1:1000 dilution of a rabbit antiserum provided by W. L. McGuire (University of Texas Health Science Center, San Antonio, TX). The latter antiserum was generated against a 17-amino acid carboxyl-terminal synthetic peptide sequence of the erbB-2 protein (20). In both cases, ^{125}I-labeled donkey anti-rabbit IgG (≈200,000 cpm/ml; Amersham) was used as a secondary antibody and receptor bands were detected by autoradiography.

For immunohistochemistry, EGF receptor and erbB-2 were assessed using the mouse monoclonal antibody 225 (26) or the anti-erbB-2 monoclonal antibody mAb1 (Triton Biosciences, Alameda, CA), respectively, both at a concentration of 10 μg/ml. Frozen sections of human placenta or slides plated with A-431 or SKBR-3 cells were used as positive controls. Sections incubated with DAKO monoclonal control antisera (DAKO) were used as negative controls. After quenching endogenous peroxidase activity, sections were rehydrated in PBS and incubated for 10 min in 10% normal goat serum in PBS containing 0.1% bovine serum albumin. After blotting off the excess of blocking serum, the primary antibody was added for an overnight incubation at 4°C. Sections were subsequently washed in PBS and incubated for 30 min each with a biotinylated goat anti-mouse IgG (Zymed Laboratories; 1:40 dilution) and peroxidase-conjugated avidin-biotin complex (DAKO). Aminoethylcarbazole was used as a chromogen as above.

RESULTS AND DISCUSSION

PLC-γ1 in Human Breast Tissues. To determine whether PLC-γ1 was expressed at different levels in malignant and nonmalignant tissues, equal amounts of tissue protein were probed with an anti-PLC-γ1 antibody. Western blot analyses (Fig. 1) revealed that in many carcinomas PLC-γ1 was readily detectable, whereas in nonmalignant tissues it was not present in sufficient amounts to be detectable in this assay. When the results in Fig. 1 were compiled with additional similar analyses, 21 of 30 (70%) carcinomas, but only 1 (a fibroadenoma) of 17 (6%) nonmalignant samples, were positive for PLC-γ1 protein. In eight cases PLC-γ1 was detectable in the carcinomas but not in adjacent normal breast tissue. Although PLC-γ1 could be detected in membrane and cytosolic fractions in several carcinomas, the predominant localization was cytosolic, particularly if the data are adjusted for the amount of membrane and cytosolic protein per cell.

To localize the cell(s) of origin of the immunodetected PLC-γ1 and to assess whether in the epithelium-scarce nonmalignant samples PLC-γ1 had been diluted, compared to the epithelium-rich carcinomas, we performed immunohistochemistry on frozen sections from 26 specimens. By this method, PLC-γ1 was readily detectable in 13 of 16 (81%) carcinomas but in only 1 (a fibroadenoma) of 10 (10%) nonmalignant samples (Fig. 2). The immunoblotting and the immunohistochemistry results agreed in 14 of 16 instances. Two carcinomas were PLC-γ1-positive by histochemistry but negative by Western blot. PLC-γ1 immunoreactivity was intense in malignant breast epithelium (Fig. 2*A*) and vascular endothelial cells but was not prominent in the stroma or in benign mammary glands (Fig. 2*C*). Similar to the examples shown in Fig. 2 *A–C*, in five cases the neoplastic mammary cells showed intense PLC-γ1 staining, but staining in the adjacent tumor-free benign breast epithelium was not detectable. There was no apparent difference in the degree of PLC-γ1 immunoreactivity between areas of infiltrating carcinoma and areas of carcinoma *in situ*. The results in Figs. 1 and 2 demonstrate that malignant mammary epithelium fre-

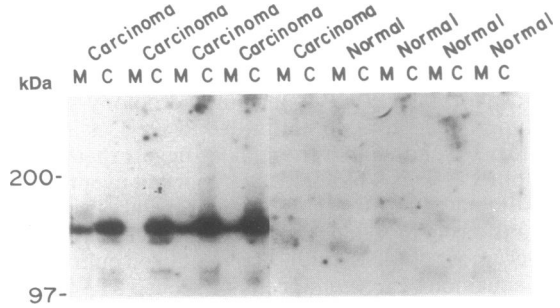

FIG. 1. Immunoblotting of membrane (M) and cytosolic (C) PLC-γ1 from human mammary tissues. Membrane and cytosolic fractions were prepared and subjected to an immunoblot procedure with a 1:500 dilution of a PLC-γ1 antiserum followed by incubation with ^{125}I-labeled donkey anti-rabbit IgG. Each lane represents 300 μg of protein. An 18-hr autoradiograph exposure is shown. Incubation of the Western blots with antiserum preincubated with immunizing peptide abolished PLC-γ1 immunodetection (not shown).

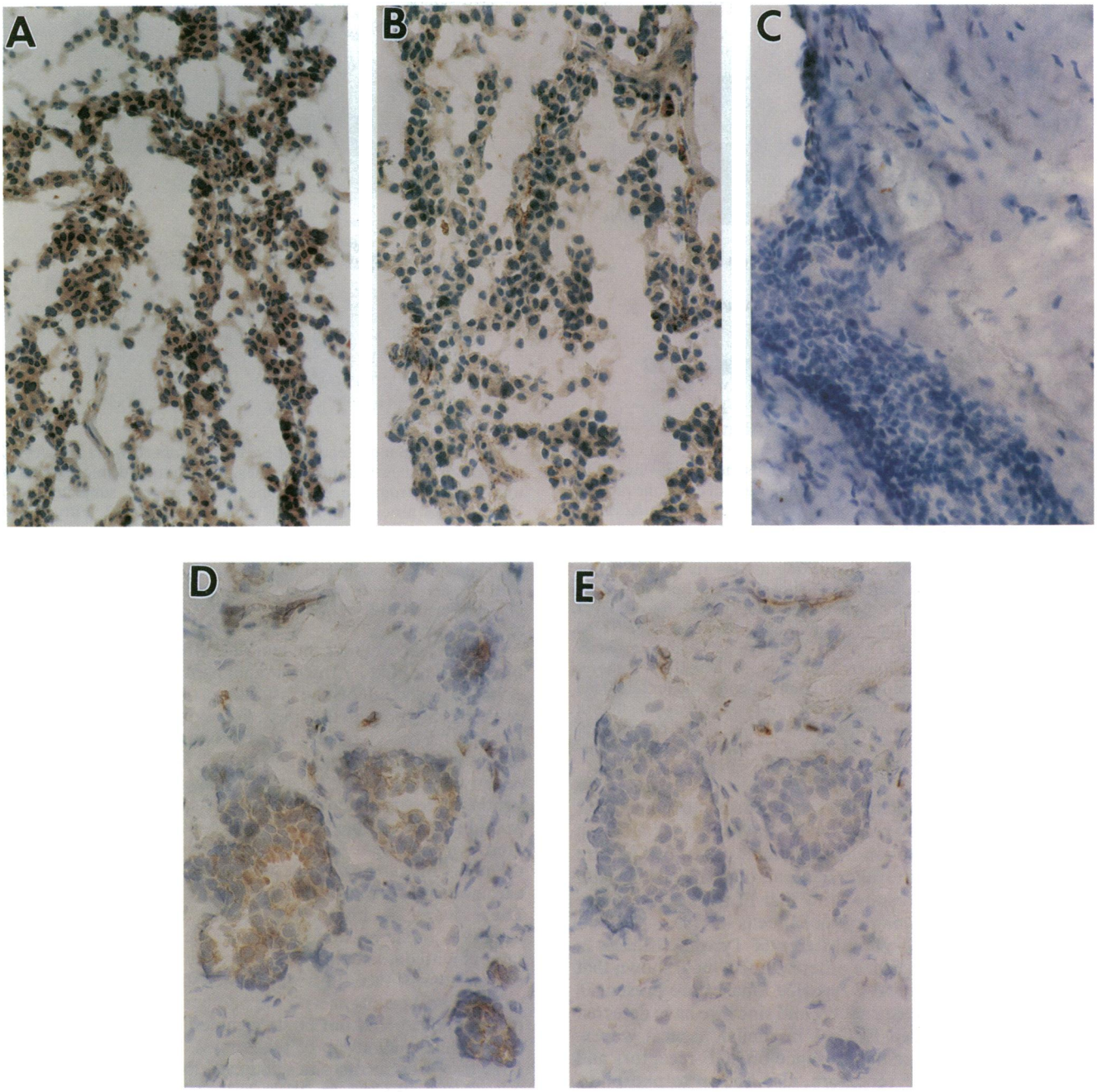

FIG. 2. PLC-γ1 immunohistochemistry in human mammary tissues. Frozen sections from an infiltrating ductal breast carcinoma (*A* and *B*), the adjacent normal breast tissue (*C*) in the same surgical specimen, and a benign fibroadenoma in a different subject (*D* and *E*) were subjected to PLC-γ1 immunohistochemistry. In *B* and *E*, frozen sections were stained with PLC-γ1 antiserum that had been preincubated with 1 mg of immunizing peptide per ml. (×145.)

quently exhibits a significantly higher content of PLC-γ1 than normal mammary epithelium.

Tyrosine Phosphorylation of PLC-γ1 *in Vivo*. Since breast carcinomas have been shown to express receptor tyrosine kinases that utilize PLC-γ1 as a substrate, we attempted to determine whether this molecule is tyrosine phosphorylated *in vivo*. The results shown in Fig. 3 demonstrate that many carcinoma tissues do contain PLC-γ1 that is retained by chromatography on an anti-phosphotyrosine column. PLC-γ1 was detectable by Western blot in the anti-phosphotyrosine eluates from 18 of the 21 (78%) PLC-γ1-positive breast cancer cytosols. In contrast, anti-phosphotyrosine adsorption of cytosolic protein from 17 nonmalignant specimens did not reveal detectable PLC-γ1. As shown in Fig. 3 *Upper*, <5% of the total cytosolic PLC-γ1 was tyrosine phosphorylated in the cancer homogeneates. This was estimated from the ratio of PLC-γ1 detected in the anti-phosphotyrosine eluates to the amount of isozyme present in the nonadsorbed fraction. The adsorption of tyrosine-phosphorylated PLC-γ1 to the anti-phosphotyrosine matrix was blocked by preincubating the matrix with excess phenylphosphate or phosphotyrosine (Fig. 3 *Lower*). Similar concentrations of phosphoserine or phosphotreonine did not block adsorption to the affinity matrix (not shown), thereby demonstrating specific adsorption. This tyrosine phosphorylated fraction is low compared to the amount of tyrosine-phosphorylated PLC-γ1 (50–70%) recovered from EGF-treated cells (2, 3, 6). It is likely, however, that rapid

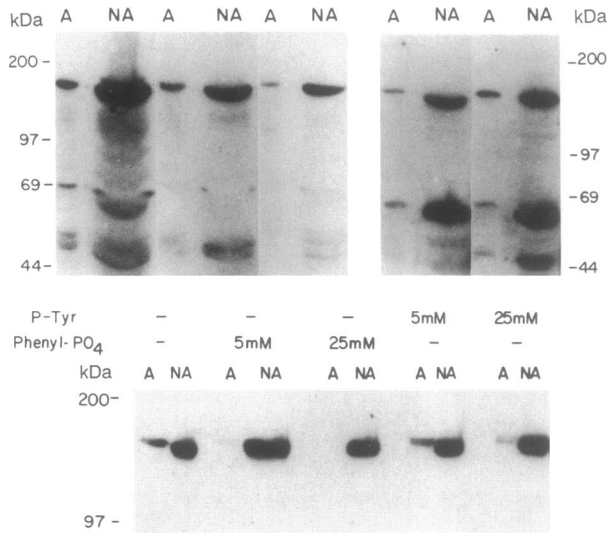

FIG. 3. (*Upper*) Presence of tyrosine-phosphorylated PLC-γ1 in breast cancer tissue. One and one-half milligrams of cytosolic protein from five invasive breast carcinomas, in a buffer containing phosphatase and protease inhibitors, was incubated with 200 μl of a Sepharose-linked anti-phosphotyrosine (monoclonal 1G2) bead matrix (23) for 2–4 hr at 4°C with rocking as described (24). After washes, the specifically adsorbed (A) phosphotyrosyl proteins were eluted with 20 mM phenylphosphate and then subjected to PLC-γ1 immunoblot. One-fifth of the respective nonadsorbed cytosol (NA) was also analyzed for PLC-γ1 to compare it to the tyrosine-phosphorylated fraction. (*Lower*) Specificity of the anti-phosphotyrosine matrix. One and one-half milligrams of cytosolic protein from a breast carcinoma was adsorbed to the anti-phosphotyrosine matrix similar to *Upper*. As indicated, this was done in the presence of excess phenylphosphate (Phenyl-PO_4, 5–25 mM) or excess phosphotyrosine (P-Tyr, 5–25 mM).

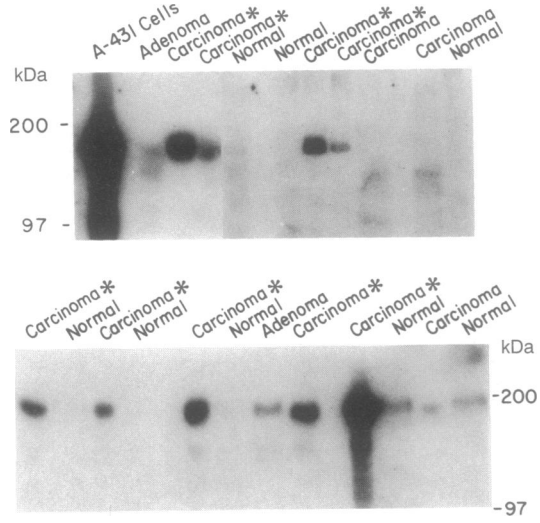

FIG. 4. Immunodetection of EGF receptors (*Upper*) and erbB-2 (*Lower*) in human mammary tissues. Two hundred micrograms of total protein from the membrane fractions was subjected to a Western blot procedure for EGF receptors or erbB-2. Fifty micrograms of membrane protein from the EGF receptor-overexpressing A-431 cells was used as control in the EGF receptor immunoblot. Eighteen-hour autoradiograph exposures are shown. Lanes marked with an asterisk are from tumors with detectable cytosolic tyrosine-phosphorylated PLC-γ1.

dephosphorylation in the tissue specimens occurred, as their handling and storage do not allow for the immediate addition of phosphatase inhibitors normally employed in cell culture experiments. Despite the inherent limitations of working with human tissues, these results show that PLC-γ1 is a tyrosine kinase substrate in human epithelial cells *in vivo*.

Coexpression of PLC-γ1 and Receptor Tyrosine Kinases in Human Breast Tissues. Since others (16–20) have shown that the EGF receptor or erbB-2 tyrosine kinases are present in high levels in some breast cancers and since we have detected tyrosine-phosphorylated PLC-γ1 in similar tissues, we examined our samples for the presence of EGF receptors and erbB-2 (Fig. 4). PDGF receptors, also known to phosphorylate PLC-γ1, are uncommon in neoplastic mammary cells (27, 28). In addition, it is unclear if fibroblast growth factor and nerve growth factor receptors are expressed in breast cancer cells and thus were not examined. All 18 carcinomas with a detectable level of tyrosine-phosphorylated PLC-γ1 contained high levels of either EGF receptors (5 of 18) or erbB-2 (16 of 18) by immunoblot analysis. In 3 PLC-γ1-positive carcinomas without detectable tyrosine-phosphorylated PLC and in 9 PLC-γ1-negative carcinomas, EGF receptors and erbB-2 were not detectable or were present in very low levels. Immunohistochemical analyses showed that in 10 of 13 carcinomas with detectable PLC-γ1, EGF receptors (1 of 10) or erbB-2 (9 of 10) were also detected. In each of these 10 cases there was a similar pattern of distribution of the PLC-γ1 staining compared to the EGF receptor or the erbB-2 staining (data not shown), suggesting colocalization of these molecules at a cellular level. Taken together, these data indicate that many breast carcinomas have the necessary elements for amplification of tyrosine kinase-dependent signal transduction systems.

It is unclear whether the marked difference in the levels of PLC-γ1 exhibited by the nonmalignant and malignant breast epithelial cells reflects the different proliferative rates of each cell type. Supporting that idea, however, is the fact that 13 of 20 (65%) PLC-γ1-positive carcinomas were also estrogen receptor (ER)-negative, whereas 7 of 9 (78%) PLC-γ1-negative tumors were ER-positive as measured by the conventional dextran-coated charcoal assay (29). It is generally accepted that ER-negative breast cancers have higher proliferative rates than ER-positive tumors (30, 31). In addition, the mean histological grade was 4.7 in the PLC-γ1-negative carcinomas and 7.7 in the PLC-γ1-positive tumors (32, 33). There were no high-grade lesions among the PLC-γ1-negative carcinomas and no low-grade lesions among those tumors with detectable PLC-γ1 protein. However, statistical correlations of PLC-γ1 with a higher stage of clinical disease (stage II vs. stage I) or with the number of metastatic axillary lymph nodes could not be determined from the characteristics of this small group of patients.

Two cell culture studies indicate that overexpression of transfected PLC-γ1 in NIH 3T3 cells does not result in cellular transformation or enhanced PDGF- or fibroblast growth factor-induced mitogenicity (34, 35). However, one preliminary report indicates that overexpression of PLC-γ1 in NIH 3T3 fibroblasts resulted in enhanced cloning efficiency in soft agar and increased tumorigenicity in athymic mice (36). Also, microinjection of anti-PLC or anti-phosphatidylinositol 4,5-bisphosphate antibodies blocked serum-induced DNA synthesis (37, 38). It remains unresolved, therefore, whether PLC activity is a critical element in the mitogenic signaling pathway. Interestingly, there is evidence suggesting that association of PLC-γ1 with the EGF receptor (ref. 39; G.C. and T. S. M. Hernandez-Sotomayor, unpublished data) or with the PDGF receptor (40) amplifies the increased activity of tyrosine-phosphorylated PLC-γ1. This would imply a stoichiometric, as well as catalytic, relationship in the activation of PLC-γ1 by receptor tyrosine kinases. Obviously, stoichiometric interactions could only be met by cells that exhibit high levels of an activating tyrosine kinase and PLC-γ1. That condition might be present in many of the breast carcinomas in this study.

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