The Role of Individual SH2 Domains in Mediating Association of Phospholipase C-γ1 with the Activated EGF Receptor*

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The two SH2 (Src homology domain 2) domains present in phospholipase C-γ1 (PLC-γ1) were assayed for their capacities to recognize the five autophosphorylation sites in the epidermal growth factor receptor. Plasma resonance and immunological techniques were employed to measure interactions between SH2 fusion proteins and phosphotyrosine-containing peptides. The N-SH2 domain recognized peptides in the order of pY1173 > pY992 > pY1068 > pY1148 > pY1086, while the C-SH2 domain recognized peptides in the order of pY992 > pY1068 > pY1148 > pY1086 and pY1173. The major autophosphorylation site, pY1173, was recognized only by the N-SH2 domain. Contributions of the N-SH2 and C-SH2 domains to the association of the intact PLC-γ1 molecule with the activated epidermal growth factor (EGF) receptor were assessed in vivo. Loss of function mutants of each SH2 domain were produced in a full-length epitope-tagged PLC-γ1. After expression of the mutants, cells were treated with EGF and association of exogenous PLC-γ1 with EGF receptors was measured. In this context the N-SH2 is the primary contributor to PLC-γ1 association with the EGF receptor. The combined results suggest an association mechanism involving the N-SH2 domain and the pY1173 autophosphorylation site as a primary event and the C-SH2 domain and the pY992 autophosphorylation site as a secondary event.

A rapid cellular response to growth factor binding to cell surface receptors is the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce two second messengers: inositol 1,4,5-trisphosphate and diacylglycerol (1). Respectively, these molecules initiate the mobilization of intracellular Ca++ and activation of protein kinase C. The mechanism by which growth factors stimulate this reaction involves the tyrosine phosphorylation-dependent activation of a specific phospholipase C (PLC) isoform: PLC-γ1 or PLC-γ2 (2, 3). Other PLC isoforms, PLCβ1–4 or PLCα1–4, are activated by growth factor-independent or unknown mechanisms. PLC-γ1 is ubiquitously expressed and targeted disruption of its gene in mice results in embryonic lethality (4). The PLC-γ2 species has a more restricted distribution (5) and has been investigated less. A PLC-γ gene has been identified in Drosophila and its disruption leads to aberrant eye development (6). In cell culture systems, however, the role of PLC-γ1 in the mitogenic response is unclear and has been described as both essential (7–13) and non-essential (14–20).

PLC-γ isoforms are structurally unique within this phospholipase family as they include Src homology (SH) domains, which enable protein/protein interactions (21, 22). PLC-γ1 contains two SH2 domains, which are 35% identical in amino acid sequence, and one SH3 domain. The SH2 domains mediate the association of PLC-γ1 with autophosphorylation sites on activated receptor tyrosine kinases (23), an essential prerequisite to PLC-γ1 tyrosine phosphorylation and activation. The function of SH3 domains is to mediate association with proline-rich sequences in partner proteins; however, the exact role of this domain in PLC-γ1 function is unclear, as no interacting protein has been convincingly identified in vivo.

In several growth factor receptors a specific autophosphorylation site is essential for PLC-γ1 association: Tyr-1021 in the platelet-derived growth factor β-receptor (19, 24–26), Tyr-766 in the fibroblast growth factor receptor (17, 18), Tyr-785 in the nerve growth factor receptor Trk (27, 28), Tyr-1169 in the vascular endothelial growth factor receptor Flt (29), Tyr-1015 in the glial-derived growth factor receptor Ret (30, 31), and Tyr-1356 of the hepatocyte growth factor receptor Met (32, 33). In each instance, mutagenesis of the essential tyrosine produces near complete abrogation of PLC-γ1 association and tyrosine phosphorylation. In the case of the epidermal growth factor (EGF) receptor, however, mutagenesis of each of the five autophosphorylation sites did not reveal a specific site essential for PLC-γ1 interaction (34). Based on the capacity of a complex of activated EGF receptor and fusion protein containing both SH2 domains of PLC-γ1 to differentially affect the sensitivity of individual phosphorylation sites to phosphatase digestion, it was found that the pY992 site was protected most while the pY1068 and pY1173 residues were protected to a lesser extent (35). Autophosphorylation sites pY1086 and pY1148 were not significantly protected. Based on these results, the authors concluded that pY992 was the major PLC-γ1 association site. However, another group demonstrated that mutagenesis of Tyr-992 to Phe had no influence on PLC-γ1 association (34) or phosphorylation (36). Hence, it remains unclear how PLC-γ1 interacts with autophosphorylation sites on the EGF receptor.

C-SH2, carboxyl-terminal SH2 domain; PBS, phosphate-buffered saline; RU, resonance units.

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The abbreviations used are: PLC, phospholipase C; EGF, epidermal growth factor; GST, glutathione S-transferase; HA, hemagglutinin; SH, Src homology; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; N-SH2, amino-terminal SH2 domain;
Since PLC-γ1 has two dissimilar SH2 domains it is also not clear whether one or both are needed for association with the EGF receptor. One analysis of SH2 fusion protein binding to activated EGF receptors showed binding by the N-SH2 domain and, based on the synergistic binding of an construct containing both N-SH2 and C-SH2 domains, concluded that the C-SH2 domain also bound to the EGF receptor (23).

To explore the mechanism of PLC-γ1 interaction with the EGF receptor in more detail we have used two approaches. First, in vitro assays have utilized surface plasma resonance spectroscopy and enzyme-linked immunosorbent assays (ELISA) to measure the binding of a panel of novel GST-SH2 constructs to phosphotyrosine-containing peptides representing each EGF receptor autophosphorylation site. The second approach employed is an *in vivo* assay in which point mutations are introduced into the PLC-γ1 molecule to disable each or both SH2 domains. The mutants were then tested in intact cells for their capacity to associate with the activated EGF receptor.

**EXPERIMENTAL PROCEDURES**

**Materials—** BIAcore streptavidin chips were obtained from BIAcore (Uppsala, Sweden). The 96-well ELISA microtiter plates (Falcon 3912) were products of Becton Dickinson Labware. The vectors pGEX-2TK and pRK5 were obtained, respectively, from Amersham Pharmacia Biotech and Dr. Alan Hall, University College, London. The ExSite polyclone reaction site-directed mutagenesis kit was purchased from Stratagene and glutathione-Sepharose beads were obtained from Amersham Pharmacia Biotech. Glutathione S-transferase (GST) monomodalclonal antibodies were purchased from Amersham Pharmacia Biotech, while antibody to phosphotyrosine and horseradish peroxidase-conjugated Protein A were from Zymed Laboratories Inc. Aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride, 2′,2′-azinois(3-ethylbenzthiazole-6-sulfonic acid), hydrogen peroxide, avidin, reagents for enhanced chemiluminescence (ECL), and Protein A-Sepharose were purchased from Sigma. Immobilon-P membranes were from Millipore. EGF was isolated from the mouse submaxillary gland according to the method of Savage and Cohen (38). The following buffers were used: Buffer A, 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA; Buffer B, 1% Triton X-100, 10% glycerol, 20 mM Heps, pH 7.2, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM Na3VO4.

**Plasmid Construction—** The rat cDNA encoding PLC-γ1 was a generous gift from Dr. Sue Goo Rhee (National Institutes of Health). A double HA epitope was added at the N terminus to generate HA-PLC-γ1R586K as N1, HA-PLC-γ1R694K as N2, and HA-PLC-γ1R586K/R694K as N1R586K/R694K as N2. Each construct was cloned into the mammalian expression vector pRK5.

GST fusion proteins were then prepared to contain the central SH2-SH2-SH3 region of PLC-γ1 (comprising residues 548–854) with the site-directed mutations at each SH2 domain described above. Also, fusion proteins containing the single N-SH2 domain (residues 548–661) or the single C-SH2 domain (residues 667–759) of PLC-γ1 were also prepared. These fusion proteins are depicted in Fig. 1A. To prepare GST constructs, primers homologous to desired sequences within the rat PLC-γ1 cDNA and containing BamHI and EcoRI restriction sites, were synthesized. The cDNA sequence encoding the indicated SH region was subsequently amplified by polymerase chain reaction using PLC-γ1 wt and the SH2 domain mutants described above as templates. The DNA was inserted into pBac2 and EcoRI, and ligated into pGEX-2TK bacterial expression vector. The fidelity of all the polymerase chain reaction-amplified fragments was verified by sequencing.

**Bacterial Expression and Protein Purification—** The recombinant GST constructs were introduced into Escherichia coli strain XL-1-blue and the bacterial transformants were analyzed for the presence of the correct insert. GST fusion proteins were then expressed following induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside and expressed fusion proteins were isolated following procedures described elsewhere (39). After purification, fusion proteins were stored at −80 °C in Buffer A supplemented with 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Protein concentrations were determined by the modified method of Bradford (Bio-Rad).

**Peptide Synthesis—** Peptides representing each of the five EGF receptor autophosphorylation sites were synthesized by Quality Controlled Biochemicals, Inc., Hopkinson, MA, as 12-mers containing a biotin cap at the N terminus which enabled attachment to streptavidin-coated CM-dextran chips for surface plasma resonance assays and to avidin-coated ELISA plates for *in vitro* peptide binding assays. The peptides were suspended in water and stored at −20 °C at a concentration of 1 mg/ml. The peptides are referred to by the Tyr residue number of the EGF receptor. The exact sequences of each peptide synthesized are as follows: pY992, ADEpYLFIPQGGF; pY1068, VPEpYINQWSPKR; pY1086, NPVPpYNQPLNPA; pY1148, NPDPypYQDFFPKE; pY1173, NAEpYLVAPQSS. One peptide, Y1173, was synthesized without phosphotyrosine, but otherwise is identical to pY1173.

**Surface Plasmon Resonance Spectroscopy—** Real time binding kinetics of PLC-γ1 SH2 fusion proteins to immobilized EGF receptor phosphotyrosine peptides were measured using a BIAcore instrument (BIAcore 2000). The BIAcore system is described elsewhere (40); but, in brief, SPR involves the use of surface plasmon resonance to monitor interactions following refractive index changes in a flow cell due to binding of molecules to an immobilized ligand. The binding of protein mass to immobilized ligand is recorded in terms of resonance units (RU; 1000 RU = 1 ng of protein bound/mm² of flow cell surface). The running buffer used in this study was PBS supplemented with 0.05% Tween 20. This buffer was also used for diluting the samples prior to injection. Streptavidin-coated CM-dextran chips were used to immobilize biotinylated peptides. Each chip contains four flow cells, one of which was left uncoupled to peptide as a control background measurement. To avoid erroneous results generated by the avidity influence of GST dimers, a low concentration of peptide (55–60 RU) was coupled to each chip, as recommended by Ladbury et al. (41).

To measure binding, the GST fusion proteins at 100, 200, 500, 400, or 500 nm concentrations were passed over the immobilized phosphotyrosine peptides or the blank chip surface at a flow rate of 10 μl/min for 10 min at 25 °C. After each binding assay, flow cells were regenerated by running 0.1% SDS (flow rate; 10 μl/min for 1 min). Regeneration did not disturb streptavidin or biotinylated peptides. To assess whether any degradation of the chip had occurred between experiments, the level of binding of GST-N-SH2-SH2-EGF was checked with a GST-N-SH2-SH2 construct of fixed concentration immediately before and after every programmed run. In all cases there was no significant change in the response. The sensogram generated by each single run was corrected by subtracting the blank response. Binding constants were then determined from the titration curves using BIAevaluation version 3.0 software. The detailed methodology for the estimation of rate constants has been described in the BIAevaluation Handbook (BIAevaluation version 3.0 software Handbook, BIAcore, Inc.).

**Peptide Binding Assay—** Wells in 96-well plates were coated with avidin (100 μg/ml in PBS) and incubated at 4 °C overnight. The wells were then filled with blocking buffer (1% BSA in PBS) and incubated for 1 h at room temperature. The plates were washed three time with PBS containing 0.05% Tween 20, tapped dry, and stored at 4 °C until use to determine the binding affinities of GST-PLC-γ1 SH2 proteins toward EGF receptor-phosphotyrosine peptides, the individual wells of avidinated plates were filled with 50 μl of a biotinylated peptide (150 nm), incubated for 1 h at room temperature, and then washed with PBS containing 0.05% Tween 20. Fusion proteins were diluted in PBS and 50 μl of each dilution (0.24 to 500 μl final concentrations) were added in triplicate to wells containing immobilized peptide. The plates were then incubated at room temperature for 1 h and washed. Horseradish peroxidase-conjugated GST antibodies, prepared as described elsewhere (42), in PBS supplemented with 0.05% Tween 20 were added into each well and incubated for an additional 1 h at room temperature. The plates were then washed six times with PBS containing 0.05% Tween 20. Bound antibodies were detected colorimetrically after adding 50 μl of a 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)/tetramethylbenzidine reagent together with 50 μl of hydrogen peroxide (H2O2) to each well. After a 20-min incubation, the absorbance readings for each well were determined at 405 nm with a Bio-Tek ELX 800 NB plate reader. In each assay, the value of a negative control containing all reagents except immobilized peptide and GST fusion protein was subtracted from individual readings.
wild-type SH2 domains are indicated as N of Bradford (Bio-Rad), using bovine serum albumin as the standard. To

measure autophosphorylation, a 25-μl aliquot of the 10-fold concentrated supernatant was added to 250 μl of Buffer B. After incubation at 37 °C overnight, the cells were transfected with 10 μl of HA antibody incubated for 30 min at 4 °C with rocking. Insoluble material was then removed by centrifugation (14,000 × g, 10 min) at 4 °C and the supernatants collected. Protein concentrations were assayed with the method of Bradford (Bio-Rad), using bovine serum albumin as the standard. To precipitate HA-PLC-γ1, approximately 35 μl of HA antibody was incubated (4 °C) overnight with 1 mg of lystate followed by a 1-h incubation with Protein A- Sepharose. Immune complexes were washed three times with Buffer B, resuspended in 1 × Laemmli buffer, and boiled for 5 min. Subsequently, samples were electrophoresed in a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes for Western blotting. Blotting was performed following procedures described elsewhere (43). The catalytic domains are indicated X and Y. The wild-type SH2 domains are indicated as N+ or C+ and mutants are N− or C−.

cells were obtained from Dr. Lee Limbird (Vanderbilt University) and were grown at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium containing 20 mM Hepes, pH 7.4, 10% calf serum, and 50 μg gentamycin.

For transient transfection of COS-M6 cells with pRK5-PLC-γ1, full-length PLC-γ1 with the same SH2 mutations in either the N- and/or C-SH2 domain. The catalytic domains are indicated X and Y. The wild-type SH2 domains are indicated as N+ or C+ and mutants are N− or C−.

Surface plasmon resonance analysis of fusion protein binding to immobilized phosphotyrosine-containing peptides, representing each of the five EGFR receptor autophosphorylation sites, was carried out using increasing concentrations of each fusion protein (100–500 nM). Representative sensograms, presented in Fig. 2, show results using 500 nM fusion protein and pY992 and pY1173 phosphopeptides. The pY992 peptide is shown as this residue has been previously implicated as a primary PLC-γ1-associated site (35), while Tyr-1173 is the major EGF receptor autophosphorylation site (48). Due to expense, we have not used a non-phosphorylated peptide to evaluate the phosphotyrosine-dependent binding of each SH2 fusion protein to each phosphorylated peptide. However, this has been done for Tyr-1173. The binding to non-phosphorylated Tyr-1173 peptide (data not shown) is the same as that shown in Fig. 2 for the blank chip. Additionally, the double SH2 domain mutant GST N+C− did not bind to any phosphotyrosine-containing peptide. Based on these facts and ample data in the literature, we conclude that the observed binding of SH2 domain fusion proteins to phosphotyrosine peptides is specific.

From the binding data obtained at each fusion protein concentration and each phosphopeptide, binding rate constants and the equilibrium association constant Ks were determined. The Ks value (4.86 × 106 M−1) for GST N−C− association with pY992 was arbitrarily set to 100% for comparative purposes. The data in Table I present the relative Ks values for each fusion protein and each Tyr(P) peptide. The GST N+C− (wild-type) fusion protein bound preferentially to pY992, pY1068, and pY1173, identifying these as major sites for PLC-γ1 interaction with the EGF receptor. The double mutant N−C− did not detectably bind to any Tyr(P) site, confirming the specificity for phosphotyrosine in this system. No fusion protein exhibited specific binding to the pY1086 peptide. Relatively low levels of fusion protein binding were detected to the peptide representing autophosphorylation site pY1148. There was, however, detectable binding for fusion proteins GST N−C− and GST C− which contain only a functional C-SH2 domain. At the pY992 and pY1068 sites stronger association also occurred with fusion proteins having a functional C-SH2 domain (GST N−C− or GST C−) compared with a functional N-SH2 domain (GST N−C− or GST N−). In contrast, association with the pY1173 autophosphorylation site required a functional N-SH2 domain (GST N−C− or GST N−) and was not detectable when the C-SH2 domain (GST N−C− or GST C−) was the only functional SH2 domain present. Hence, the pY1173 site, which is the major autophosphorylation site in vivo (48), demonstrates a high level of selectivity for interaction with the N-SH2 domain of PLC-γ1.

Fig. 1. Schematic representations of SH2 domain fusion proteins and PLC-γ1 mutants. Panel A, GST fusion proteins containing the entire SH region (SH2-SH2-SH3) of PLC-γ1 and having, as indicated, Arg to Lys mutations. Fusion proteins with the single N-SH2 or COOH-terminal SH2 domains are also shown. Panel B, HA-tagged constructs, 5 × 106 cells were plated in a 100-mm culture dish and incubated at 37 °C overnight. The cells were transfected with 1 μg of each construct using the DEAE method described elsewhere (43). The transfected cells were subsequently incubated for 48 h at 37 °C in complete media. Prior to EGF addition, the cells were incubated overnight in media containing 0.5% serum. The cells were then treated with EGF (100 ng/ml) for 1 h at 4 °C.

Immunoprecipitation and Western Blotting—After EGF treatment, the cells were washed three times with ice-cold Ca2+- and Mg2+-free PBS and lysed with 300 μl of Buffer B. After scraping, the lysates were incubated for 30 min at 4 °C with rocking. Insoluble material was then removed by centrifugation (14,000 × g, 10 min) at 4 °C and the supernatants collected. Protein concentrations were assayed with the method of Bradford (Bio-Rad), using bovine serum albumin as the standard. To precipitate HA-PLC-γ1, approximately 35 μl of HA antibody was incubated (4 °C) overnight with 1 mg of lystate followed by a 1-h incubation with Protein A-Sepharose. Immune complexes were washed three times with Buffer B, resuspended in 1 × Laemmli buffer, and boiled for 5 min. Subsequently, samples were electrophoresed in a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes for Western blotting. Blotting was performed following procedures described elsewhere (44). Bound antibody was detected by ECL.

RESULTS

Surface Plasmon Resonance Analysis—In most published studies of SH2 interaction with phosphotyrosine peptide, individual SH2 domain fusion proteins have been employed. However, to assess the function of PLC-γ1 SH2 domains within a larger segment of the native protein, we constructed the fusion proteins shown in Fig. 1A to contain the entire central region of PLC-γ1, which contains two SH2 domains and one SH3 domain. Hence, this fusion protein includes residues 548–854 of the native molecule and represents approximately 25% of the total sequence. In these SH2 domains, mutation of a conserved Arg residue within the FLVRES consensus sequence of SH2 domains (45) was introduced as indicated (Fig. 1A), to disable one or both SH2 domains. Mutation of this conserved Arg residue to Lys has been demonstrated in other SH2 domains to ablate phosphotyrosine binding (46, 47). For comparison, we also produced and analyzed fusion proteins containing only one SH2 domain, as depicted in Fig. 1A.

ELISA Assay of SH2 Domain Binding—The data obtained with surface plasmon resonance suggest distinct patterns of
selectivity of the N-SH2 and C-SH2 domains of PLC-γ1 for EGF receptor autophosphorylation sites, particularly pY1173, the major in vivo autophosphorylation site. To test these apparent selectivities, we employed an alternative assay that avoids some of the intricacies of surface plasmon resonance. Biotinylated peptides containing phosphotyrosine corresponding to Tyr-992 and Tyr-1173 of the EGF receptor were coupled to streptavidin-coated sensor surface as described under “Experimental Procedures.” The indicated GST fusion proteins (500 nM) were then passed over the chip and the real time binding response plotted as resonance unit signals (RU) relative to time. In each plot, solid and broken lines, respectively, indicate interactions of each fusion protein with the blank surface and phosphorylated peptides. Panels A, C, E, and G represent binding response of the indicated fusion proteins to pY992 or the blank surface, while Panels B, D, F, and H show binding to Tyr(P) 1173 or the blank surface, respectively.

also obtained by surface plasmon resonance. Also, similar to surface plasmon resonance data, the double mutant (GST N’ C’) did not associate with any Tyr(P) peptide and only fusion proteins having a functional C-SH2 domain (GST N’ C’, GST N’ C’, and GST C’) demonstrated binding to the pY1148 peptide. Similarly, only fusion proteins with a functional N-SH2 domain (GST N’ C’, GST N’ C’, and GST N’) recognized the pY1173 peptide. The pY992 and pY1068 peptides showed capacities to associate with fusion proteins having either an N-SH2 or C-SH2 domain, although in both cases a functional C-SH2 domain bound more effectively than a functional N-SH2 domain. In summary, this analysis reflects the order of preferences observed with the BIAcore surface plasmon resonance.

Analysis of PLC-γ SH2 Domains in Intact Cells—The data obtained above in surface plasmon resonance and ELISA assays are, in general, consistent with each other in regard to the selectivity of PLC-γ1 N-SH2 and C-SH2 domains for individual
TABLE I
Relative binding of SH2 domain constructs to EGF receptor autophosphorylation site peptides

Surface plasmon resonance data for each fusion protein (Fig. 1A) and each autophosphorylation site peptide were used to calculate rate constants and the equilibrium association constant \( K_a \). The \( K_a \) value for GST \( N^1 \) interaction with the pY992 peptide was arbitrarily set to 100% and other \( K_a \) values for each indicated association expressed relative to this value.

| Peptide   | GST N^1  | GST N^C  | GST N^1  | GST N^C  | GST N^1  | GST N^C  |
|-----------|----------|----------|----------|----------|----------|----------|
| pY992     | 100^a    | 34       | 82       | ND^b     | 40       | 439      |
| pY1068    | 55       | 11       | 32       | ND       | 21       | 239      |
| pY1148    | ND       | ND       | ND       | ND       | ND       | ND       |
| pY1173    | 6        | 7        | 25       | ND       | 104      |          |

^a \( K_a = 4.86 \times 10^8 \text{ M}^{-1} \)
^b ND, not detectable.

**Fig. 3.** ELISA assay of PLC-\( \gamma \)1 SH domain fusion protein binding to EGF receptor phosphotyrosine peptides. The binding of fusion proteins with peptides corresponding to the indicated EGF receptor phosphotyrosine residues were analyzed using an ELISA based assay, as described under “Experimental Procedures.” Panels A, B, C, D, and E represent the binding responses of fusion proteins representing pY992, pY1068, pY1086, pY1148, and pY1173, respectively. Panel F showed the binding of fusion proteins to the blank surface. Each point in the plots represent the mean of triplicate measurements. The open square [■] curves represent peptide binding by GST \( N^1 \), the closed square [■] by GST \( N^C \), the open circle [○] by GST \( N^1 \), the closed circle [●] by GST \( N^C \), the open triangle [▲] by GST \( N^1 \), and the closed triangle [▼] by GST \( N^C \).

EGF receptor autophosphorylation sites. However, the in vitro data employ fusion proteins that may or may not reflect the behavior of SH2 domains within the context of the entire PLC-\( \gamma \)1 molecule. Therefore, we have used the same Arg to Lys mutations of SH2 domains within the context of the entire PLC-\( \gamma \)1 molecule to examine SH2 function in the holoenzyme. Also, the in vitro assays consider only two reactants and assume concentrations, for example, an equivalent stoichiometry for each autophosphorylation site, that may not be relevant to the same reaction in the intact cell. To examine the influence of these mutations on PLC-\( \gamma \)1 function in vivo, mutant and wild-type PLC-\( \gamma \)1 molecules were transiently expressed in COS cells and following the addition of EGF, the co-precipitation of EGF receptors with HA-tagged PLC-\( \gamma \)1 isoforms was assessed. The results are shown in Fig. 5A. In parallel, an equal aliquot of each lysate was also precipitated with anti-HA and blotted with anti-phosphotyrosine to measure the extent of PLC-\( \gamma \)1 tyrosine phosphorylation (Fig. 5B). To assess the level of HA-PLC-\( \gamma \)1 present in the lysates, the blot shown in Fig. 5B was stripped and re-probed with anti-HA, as shown in Fig. 5C. The data in Fig. 5C was then used to normalize receptor association data (Fig. 5A) and PLC-\( \gamma \)1 tyrosine phosphorylation data (Fig. 5B). These normalized and quantitated results are presented in Fig. 6.

In regard to EGF receptor association, only PLC-\( \gamma \)1 molecules having a functional N-SH2 domain (N\(^{C^+}\) and N\(^{C^-}\)) displayed a measurable capacity to form an association complex with activated EGF receptors (Fig. 6A). Complex formation with the N\(^{C^-}\) mutant was approximately 30% of that recorded with the wild-type (N\(^{C^+}\)) PLC-\( \gamma \)1 molecule and, in contrast, the N\(^{C^-}\) mutant, having only a functional C-SH2 domain, was only 1% as effective as wild-type PLC-\( \gamma \)1 in formation of a complex with the activated EGF receptor. The activity of this latter mutant was only slightly above that of the N\(^{C^-}\) double mutant.

When the different mutants were compared for their levels of tyrosine phosphorylation, the same rank order was obtained. Under the “equilibrium” conditions of this assay, i.e. 40-min incubation at 4 °C, the N\(^{C^-}\) mutant was tyrosine phosphorylated to approximately the same extent (97%) as the wild-type molecule. The small level of receptor association observed with the N\(^{C^-}\) mutant was paralleled by a measurable level of tyrosine phosphorylation equal to about 12% of that recorded with the wild-type PLC-\( \gamma \)1. Comparison of these results to the
significantly more reduced association capacities of the same mutants (Fig. 6A) indicates that, under these conditions, the tyrosine-phosphorylated species of PLC-γ1 is more metabolically stable than the PLC-γ1-EGF receptor complexes. In the absence of any functional SH2 domain (N-'), no detectable PLC-γ1 receptor association or tyrosine phosphorylation was observed.

**DISCUSSION**

The data in this paper lead to the conclusion that PLC-γ1 interacts with the activated EGF receptor by a mechanism that involves the N-SH2 domain as a primary association event and the C-SH2 domain as a secondary event necessary for a maximal level of association. Together with other published data this model would suggest that the N-SH2 domain interacts primarily with pY1173, which is the major EGF receptor autophosphorylation site in vivo (48). The C-SH2 domain of PLC-γ1 most likely interacts with pY992, a minor autophosphorylation site in vivo (49).

As reported elsewhere (35), phosphatase protection experiments in vitro showed that a fusion protein containing the single C-SH2 domain of PLC-γ1 blocked dephosphorylation of pY992 and a fusion protein having both N-SH2 and C-SH2 domains blocked dephosphorylation of the pY1068 and pY1173 sites in addition to pY992. The single N-SH2 domain of PLC-γ1 was not assessed in that study. Hence, our data and conclusions are not in disagreement with the phosphatase protection data, but rather emphasize the contribution of the N-SH2 domain and the pY1173 site.

The model described above is also consistent with published mutagenesis data. Analysis of single site autophosphorylation mutants showed that the Y1173F mutation decreased wild-type PLC-γ1 association by 35%, while no decrease was observed with the Y992F mutant (34). The Y1173F mutant also had a decreased capacity (~30%) to produce inositol phosphates following the addition of EGF (50). Since our data show that only the N-SH2 domain mediates PLC-γ1 association with the EGF receptor in cells, it seems reasonable to conclude that the PLC-γ1 activation and association with the Y1173F mutant observed in these prior studies were mediated by interactions between the N-SH2 domain and other autophosphorylation sites, such as pY1068.

Analyses of carboxyl-terminal deletion mutants of EGF receptor also support a major role for the pY1173 site in mediating PLC-γ1 association. Deletion of residues encompassing the pY1173 and pY1148 sites substantially decreased binding to the EGF receptor (51). In a separate study, deletion of Tyr(P) sites 1173 and 1148 dramatically decreased binding to the EGF receptor of a fusion protein containing both SH2 domains of PLC-γ1 (52). Similar results implicating carboxyl-terminal autophosphorylation sites in the control of PLC-γ1 interaction with the EGF receptor have been reported for the multiple autophosphorylation site mutants Y1173F,Y1148F and Y1173F,Y1148F,Y1068 sites (34). In summary, available mutagenesis studies are all consistent with a major role of pY1173 in mediating PLC-γ1 association with the EGF receptor and a lesser role for pY992. Unfortunately, stoichiometry data re-

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**Fig. 5.** EGF receptor association with the wild-type and SH2 domain mutants of PLC-γ1. COS-M6 cells were plated in a 100-mm dish and the next day the cells were transfected with different PLC-γ1 constructs as described under “Experimental Procedures.” The cells were then incubated at 37 °C for 48 h in complete media. After overnight incubation in media containing 0.5% serum, the cells were treated with Buffer B and the cell extracts were subjected to immunoprecipitation. Panel A, a 1-mg aliquot of lysate was immunoprecipitated with HA antibodies and analyzed by Western blotting with EGF receptor antibodies. Panel B, similarly, an aliquot (1 mg) of lysate was immunoprecipitated with HA antibodies and Western blotted with anti-phosphotyrosine. Panel C, the phosphotyrosine blot was stripped and reprobed with HA antibodies. In each case bound antibodies were detected by ECL.

**Fig. 6.** Quantitation of PLC-γ1 association with the EGF receptor and PLC-γ1 tyrosine phosphorylation. The Western blot data shown in Fig. 5 were quantitated using a Bio-Rad imaging densitometer and molecular analyst software. The values obtained for PLC-γ1 association with the EGF receptor in Panel A and the tyrosine phosphorylation of PLC-γ1 in Panel B were normalized to the expression level of PLC-γ1, as shown in Panel C. The values for the wild-type (N-') PLC-γ1 were set to 100% and the values for SH2 mutants are expressed relative to this value.
Phospholipase C-γ1

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The phosphorylation of these two autophosphorylation sites in vivo is not available. Hence, it is not possible to assess their relative contributions.

Based on the screening of degenerate peptide libraries with various SH2 domains, consensus recognition sites for each SH2 domain have been developed (53). These data indicated that the N-SH2 domain of PLC-γ1 would have a preference for a pYLEL site and it was suggested that the N-SH2 domain might recognize the pYLRV sequence at pY1173 in the EGF receptor. This is directly supported by our data and model. In the studies with peptide libraries (53), the C-SH2 domain of PLC-γ1 showed a selectivity for a pVYIIP site which compares favorably with the pYLIP sequence at pY992. In fact, the same studies reported that, following Val or Ile at the +1 position, the C-SH2 domain of PLC-γ1 recognized Leu most frequently. The sequences at other EGF receptor autophosphorylation sites (pY1068, pY1068, and pY1148) do not conform to consens...