Inhibition of binding of phospholipase Cγ1 SH2 domains to phosphorylated epidermal growth factor receptor by phosphorylated peptides

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A series of tyrosine-containing peptides 1–12:

Asp-Ala-Asp-Glu-Tyr^{992}(PO$_3$H$_2$)-Leu-Ile-Pro-Gln-Gln-Gly-OH (1)
Asp-Ala-Asp-Glu-Tyr^{992} -Leu-Ile-Pro-Gln-Gln-Gly-OH (2)
Phe-Leu-Pro-Val-Pro-Glu-Tyr^{1068}(PO$_3$H$_2$)-Ile-Asn-Gln-Ser-Val-OH (3)
Phe-Leu-Pro-Val-Pro-Glu-Tyr^{1068} -Ile-Asn-Gln-Ser-Val-OH (4)
Asp-Asn-Pro-Asp-Tyr^{1148}(PO$_3$H$_2$)-Gln-Gln-Asp-Phe-Phe-OH (5)
Asp-Asn-Pro-Asp-Tyr^{1148} -Gln-Gln-Asp-Phe-Phe-OH (6)
Ala-Glu-Tyr^{1173}(PO$_3$H$_2$)-Leu-Arg-Val-Ala-Pro-Gln-Ser-OH (7)
Ala-Glu-Tyr^{1173} -Leu-Arg-Val-Ala-Pro-Gln-Ser-OH (8)
Ala-Glu-Tyr^{1173}(PO$_3$H$_2$)-Leu-Arg-Val-Ala-OH (9)
Ala-Glu-Tyr^{1173} -Leu-Arg-Val-Ala-OH (10)
Tyr^{1173}(PO$_3$H$_2$)-Leu-Arg-Val-Ala-Pro-Gln-Ser-OH (11)
Tyr^{1173} -Leu-Arg-Val-Ala-Pro-Gln-Ser-OH (12)

(six pairs with and without the tyrosine phosphorylated) has been synthesized. The peptides were derived from tyrosine autophosphorylation sites in the epidermal growth factor receptor (EGFR): Tyr 992, 1068, 1148 and 1173. Peptide 1, derived from the Tyr 992 site, inhibited binding of a ^{35}S-labelled fusion protein containing both of the SH2 domains from PLCγ1 to the phosphorylated EGFR with an IC$_{50}$ of 8 μM. All of the phosphorylated peptides except 11 (1, 3, 5, 7 and 9) inhibited this binding to some degree (20–55%) at 10 μM. The nonphosphorylated peptides were inactive in this assay. The nonphosphorylated peptides 2, 4, 6, 8, 10 and 12 were obtained by standard solid-phase synthetic methodologies using both Boc/benzyl and Fmoc/tert-butyl strategies. The phosphorylated peptides 1, 3, 5, 7, 9 and 11 were similarly obtained using a Fmoc/tert-butyl strategy incorporating unprotected N^x-Fmoc-Tyr, followed by phosphitylation and oxidation of the tyrosine in the resin-bound peptide. In addition, Asp-Ala-Asp-Glu-Phe^{992}(4-CH$_2$PO$_3$H$_2$)-Leu-Ile-Pro-Gln-Gln-Gly-OH (15), an analog of 1 incorporating an enzymatically stable phosphotyrosine mimic, 4-phosphonomethyl-L-phenylalanine, was synthesized and found to be inactive. © Munksgaard 1993.

Key words: epidermal growth factor receptor; inhibition; phospholipase Cγ1; phosphorylated peptides; tyrosine phosphorylation; src homology 2

The binding of the epidermal growth factor (EGF) to its receptor (EGFR) initiates a complex series of intracellular events which ultimately culminates in mitogenesis (1). An essential element in the transduction of this mitogenic signal is the ability of the EGFR to phosphorylate specifically tyrosine residues in substrate proteins (2, 3). Phospholipase Cγ1 (PLCγ1) is an established, biologically relevant substrate for the EGFR (4). Its phosphorylation by the EGFR is dependent upon the initial phosphorylation of five tyrosines (Tyr 992, 1068, 1086, 1148 and 1173) in the noncatalytic carboxylic acid (C) terminus of the receptor (5). Prior to its phosphorylation, PLCγ1 binds to the phosphotyrosine-containing C-terminus of the EGFR, thereby facilitating its phosphorylation (6). The region of PLCγ1 involved in this binding contains two src homology 2 (SH2) domains, which are highly conserved, noncatalytic sequences of approximately 100 amino acids known to bind specifically to sequences containing phosphotyrosine (7, 8).

In an attempt to disrupt the signal transduction pathway involving the phosphorylation of PLCγ1 by the EGFR, we postulated that it should be possible to inhibit specifically their SH2-mediated association with small tyrosine-phosphorylated peptides derived from the autophosphorylated tyrosines in the EGFR. A similar strategy has previously been applied to the platelet-derived growth factor receptor–phosphatidylinositol 3-kinase mediated signal transduction pathway (9). Here we report the synthesis and evaluation of a series of peptides 1–12 (Table 1) as inhibitors of PLCγ1 SH2 domain binding to the phosphorylated EGFR as determined in a new quantitative assay recently reported by us (10). The peptides include both phosphorylated (1, 3, 5, 7, 9, 11) and nonphosphorylated (2, 4, 6, 8, 10, 12) analogs derived from tyrosines autophosphorylated in the EGFR (Tyr 992, 1068, 1148 and 1173). We have identified peptide 1, derived from Tyr 992, as the first

potent inhibitor of this binding. We also report the synthesis and inactivity of 15 (and its dimethyl ester precursor 13), an analog of 1 incorporating an enzymatically stable phosphotyrosine mimic, 4-phosphono-methyl-L-phenylalanine (Pmp).

CHEMISTRY

The nonphosphorylated peptides 2, 4, 6, 8, 10 and 12 in Table 1 were prepared by standard solid-phase synthetic methodologies (11) on an Applied Biosystems (ABI) 431A peptide synthesizer. Thus, 2, 4, 10 and 12 were assembled on 4-hydroxymethylphenoxymethyl (HMP) polystyrene resin using a 9-fluorenylmethoxycarbonyl/tert-butyl (Fmoc/t-Bu) strategy with a 1-hydroxybenzotriazole/dicyclohexylcarbodiimide (HOBT/DCC) mediated coupling scheme in N-methylpyrrolidinone (NMP) with the two following deviations from the standard. First, the tyrosine was incorporated with its phenolic moiety unprotected, so as to render it available for subsequent phosphorylation on the resin. Second, the terminal amino acid was incorporated as its N^{α}-tert-butyloxycarbonyl (Boc) derivative such that cleavage of this group could be effected concomitantly with cleavage of the peptide from the resin rather than in a separate step. This allowed each peptide and its phosphorylated counterpart to be obtained from the same machine synthesis. These peptides were cleaved from the resin, and the protecting groups removed, by treatment with trifluoroacetic acid (TFA) and various scavengers (12). Peptides 6 and 8 were assembled on phenylacetamidomethyl (PAM) polystyrene resin using a Boc/benzyl (Bzl) strategy with an HOBT/DCC-mediated coupling scheme in NMP. These peptides were cleaved from the resin, and the protecting groups removed, by treatment with hydrogen fluoride (HF)/anisole.

Phosphorylated peptides 1, 3, 5, 7, 9 and 11 in Table 1,

TABLE 1

Synthetic peptides derived from EGFR

No.	Sequence (Tyr from EGFR labelled)
1	Asp-Ala-Asp-Glu-Tyr^{992}(PO$_3$H$_2$)-Leu-Ile-Pro-Gln-Gln-Gly-OH
2	Asp-Ala-Asp-Glu-Tyr^{992} -Leu-Ile-Pro-Gln-Gln-Gly-OH
3	Phe-Leu-Pro-Val-Pro-Glu-Tyr^{1068}(PO$_3$H$_2$)-Ile-Asn-Gln-Ser-Val-OH
4	Phe-Leu-Pro-Val-Pro-Glu-Tyr^{1068} -Ile-Asn-Gln-Ser-Val-OH
5	Asp-Asn-Pro-Asp-Tyr^{1148}(PO$_3$H$_2$)-Gln-Gln-Asp-Phe-Phe-OH
6	Asp-Asn-Pro-Asp-Tyr^{1148} -Gln-Gln-Asp-Phe-Phe-OH
7	Ala-Glu-Tyr^{1173}(PO$_3$H$_2$)-Leu-Arg-Val-Ala-Pro-Gln-Ser-OH
8	Ala-Glu-Tyr^{1173} -Leu-Arg-Val-Ala-Pro-Gln-Ser-OH
9	Ala-Glu-Tyr^{1173}(PO$_3$H$_2$)-Leu-Arg-Val-Ala-OH
10	Ala-Glu-Tyr^{1173} -Leu-Arg-Val-Ala-OH
11	Tyr^{1173}(PO$_3$H$_2$)-Leu-Arg-Val-Ala-Pro-Gln-Ser-OH
12	Tyr^{1173} -Leu-Arg-Val-Ala-Pro-Gln-Ser-OH
13	Asp-Ala-Asp-Glu-Phe^{992}[4-CH$_2$PO(OCH$_3$)$_2$]-Leu-Ile-Pro-Gln-Gln-Gly-OH
15	Asp-Ala-Asp-Glu-Phe^{992}(4-CH$_2$PO$_3$H$_2$) -Leu-Ile-Pro-Gln-Gln-Gly-OH

the analogous phosphorylated tyrosine derivatives, were prepared according to a recently reported on-the-resin phosphorylation procedure (13). These peptides were first assembled in nonphosphorylated form incorporating unprotected N^{α}-Fmoc-Tyr on HMP resin using the Fmoc/t-Bu strategy described above. Phosphitylation of the resin-bound peptide containing the unprotected tyrosine hydroxy group with di-t-Bu-N,N-diethylphosphoramidite and tetrazole in anhydrous tetrahydrofuran (THF), and subsequent oxidation with aqueous t-Bu hydroperoxide in dichloromethane (DCM) according to the prescribed procedure with the minor modifications discussed below, followed by cleavage and deprotection with TFA and scavengers, furnished the phosphorylated peptides as the only major product. Little of the corresponding nonphosphorylated peptides was obtained (HPLC).

Peptides **1–12** were all isolated in good yields by preparative C-18 reversed-phase high-performance liquid chromatography (HPLC). All peptides were > 95% pure by analytical HPLC, which was confirmed by capillary-zone electrophoresis (CZE) (except **2**, which was only 83% pure by CZE, and **5**, which did not elute under the conditions used). The phosphorylated peptides **1, 3, 5, 7, 9** and **11** were well separated and faster eluting than their nonphosphorylated analogs **2, 4, 6, 8, 10** and **12** in HPLC, while they were also well separated but slower eluting in CZE using an acidic buffer. All peptides exhibited amino acid analyses, nuclear magnetic resonance (NMR) spectra, and mass spectra (MS) supportive of their structures. In the ^{1}H-NMR spectra, the doublet due to the aromatic protons *ortho* to the tyrosine hydroxy group was shifted downfield 0.29–0.43 ppm in its phosphorylated analog, while that of the protons *meta* was also shifted downfield, but only 0.06–0.15 ppm. This deshielding indicated that phosphorylation had indeed occurred on the tyrosine hydroxy group.

The originally reported phosphorylation conditions (13) of phosphitylation using 10 equiv. of di-t-Bu-N,N-diethylphosphoramidite and 30 equiv. of tetrazole in THF (not specifically dried), followed by oxidation with 20 equiv. of 70% aqueous t-Bu hydroperoxide in DCM initially did not work well in our hands. When these conditions were first applied to the synthesis of **7**, the ratio (determined by HPLC analysis of the crude product) of the desired phosphorylated **7** to the undesired nonphosphorylated **8** was only 25/75. When the same conditions were applied to the synthesis of **5**, the ratio of the desired phosphorylated **5** to the undesired nonphosphorylated **6** was 55/45. When N,N-dimethylformamide (DMF) (ABI, peptide synthesis grade, dried over 4 Å molecular sieves) was substituted for THF and DCM in the original procedure in the synthesis of 7, only the nonphosphorylated **8** was observed. When these same conditions were applied to the synthesis of 5, the ratio of phosphorylated **5** to nonphosphorylated **6** was 7/93. When NMP (ABI, peptide synthesis grade)

was substituted for THF in the original procedure in the synthesis of **7**, the ratio of phosphorylated **7** to nonphosphorylated **8** was 2/98. This problem was resolved when the equivalents of di-t-Bu-N,N-diethylphosphoramidite and tetrazole were increased to 50 and 150, respectively, and anhydrous THF was used. When these new conditions were applied to the synthesis of **7**, the ratio of phosphorylated **7** to nonphosphorylated **8** was 97/3. Similarly, these new conditions applied to the synthesis of **5** gave a ratio of phosphorylated **5** to nonphosphorylated **6** of 87/13. We concluded that the phosphitylation reaction was quite water-sensitive.

The dimethylphosphonate-containing peptide **13** was assembled on PAM resin using a Boc/Bzl strategy with an HOBT/DCC-mediated coupling scheme in NMP. The N^{α}-Boc-4-dimethylphosphonomethyl-L-phenylalanine which was incorporated was prepared by a recently described asymmetric synthesis (14). This peptide was cleaved from the resin and the protecting groups removed by treatment with HF/p-cresol, leaving the dimethyl esters of the phosphonic acid intact. In addition to the desired **13**, a byproduct whose structure was determined to be acetyl-Leu-Ile-Pro-Gln-Gln-Gly-OH (**14**) was isolated. This obviously resulted from incomplete coupling of the N^{α}-Boc-Pmp derivative to the leucine under the standard conditions of single coupling with four equivalents of the amino acid employed. Treatment of **13** with trifluoromethanesulfonic acid (TFMSA)/TFA/dimethyl sulfide (DMS)/m-cresol (10:50:30:10, v/v) as described (14) removed the dimethyl esters to give the desired **15**. This reaction also yielded a byproduct whose structure was not determined, but whose MS indicated that H_2O had been eliminated from **15**. Peptides **13** and **15** were also isolated by preparative HPLC and were > 95% pure by analytical HPLC. These peptides were 90 and 100% pure, respectively, by CZE, and exhibited amino acid analyses, NMR spectra and MS supportive of their structures.

BIOLOGY

The assay employed to study the inhibitory properties of the synthetic peptides specifically measures the association between the SH2 domains of PLCγ1 with the phosphorylated EGFR, and has recently been described (10). Briefly, a bacterial glutathione-S-transferase fusion protein, containing both SH2 domains of PLCγ1, was labelled *in vivo* with ^{35}S. This protein bound to the purified EGFR (complexed with protein A–Sepharose beads) from EGF-treated cells, whereas no binding was detected in nontreated cells, thus demonstrating an absolute requirement for phosphorylated receptor. This binding was rapid, reaching apparent equilibrium within 10 min. Inhibition experiments were performed under apparent equilibrium binding conditions in which the ^{35}S-labelled SH2 fusion protein was incubated with the phosphorylated EGFR–beads com-

plex in the presence of increasing concentrations of the peptides **1–12**, **13**, **15**. All of the phosphorylated peptides except **11** (**1**, **3**, **5**, **7**, **9**) significantly inhibited (20–55%) the binding of the SH2 fusion protein to the phosphorylated EGFR at 10 μM. These results are reported in Fig. 1 as the percentage of maximal binding of the ^{35}S-labelled SH2 fusion protein to the phosphorylated EGFR–beads complex vs. peptide concentration. The nonphosphorylated peptides **2**, **4**, **6**, **8**, **10** and **12**, as well as the Pmp analogs **13** and **15**, did not inhibit this binding even at 1 mM (data not shown). Peptide **1**, a phosphorylated 11 amino acid peptide encompassing Tyr 992, was the most potent inhibitor, with an IC$_{50}$ of 8 μM.

DISCUSSION

The most important point to be made concerning the activity of the peptides **1–12** in inhibiting the binding of the SH2 fusion protein to the phosphorylated EGFR is the absolute requirement for a phosphorylated tyrosine. Phosphorylated peptides **1**, **3**, **5**, **7** and **9** inhibited binding in this assay 20–55% at 10 μM, whereas all unphosphorylated peptides (**2**, **4**, **6**, **8**, **10**, **12**) were without activity even at 1 mM. The stringency of this requirement is highlighted by the loss of activity when the phosphate moiety in **1** was changed to a phosphonomethyl moiety in **15**. These results are consistent with the explanation that these phosphorylated peptides mimic phosphorylated segments of the C-terminus of the EGFR and compete with it for the SH2 domains of PLCγ1.

Secondly, the importance of the residues adjacent to the phosphorylated tyrosine residue in the peptides is emphasized by considering the inhibitory activity of the

three peptides encompassing Tyr 1173, peptides **7**, **9** and **11**. When the three C-terminal amino acids of **7** were deleted to give **9**, the inhibitory activity increased. However, when the two N-terminal amino acids of **7** were deleted to give **11**, inhibitory activity was abolished. Thus, some amino acids surrounding the phosphorylated tyrosine in these peptides are necessary for significant inhibitory activity, while others appear to be deleterious.

Thirdly, although phosphorylated peptides representing Tyr 992, 1068, 1148 and 1173 of the EGFR were all significantly active in this assay, there were clear differences. Peptide **1**, derived from Tyr 992, was the most potent with an IC$_{50}$ of 8 μM. The second most potent peptide was that derived from Tyr 1068, peptide **3**, with an IC$_{50}$ of 26 μM. It is very interesting to note that in recent studies examining the protection of EGFR dephosphorylation by PLCγ1 SH2 fusion proteins, Tyr 992 was in fact demonstrated to be the highest affinity binding site on the EGFR for the SH2 domains of PLCγ1, followed by Tyr 1068 (15). We are presently engaged in efforts to optimize the inhibitory activity of these peptides in this binding assay.

EXPERIMENTAL PROCEDURES

Materials

All amino acids are of the L configuration. Purchased reagents were obtained from the following sources: N^{α}-Fmoc-Tyr, Bachem Bioscience; N^{α}-Boc-Tyr, Bachem California; all other N^{α}-Fmoc and N^{α}-Boc-amino acids, ABI or Bachem California; HMP, N^{α}-Boc-Phe-PAM, N^{α}-Boc-Gly-PAM, and N^{α}-Boc-Ser(Bzl)-PAM polystyrene resins, ABI; piperidine, 4-dimethylaminopyridine (DMAP), methanol (MeOH), HOBT, DCC, diisopropylethylamine (DIEA), acetic anhydride, TFMSA, dimethyl sulfoxide (DMSO), DMF, DCM and NMP, ABI; DMF, DCM and NMP, Burdick and Jackson (high-purity grade); anhydrous THF, tetrazole, 1,2-ethanedithiol (EDT), anisole, DMS, thioanisole, m-cresol, p-cresol, phenol, acetic acid (AcOH) and 70% aqueous t-Bu hydroperoxide, Aldrich Chemical; TFA, ABI and Halocarbon Products; H$_2$O and CH$_3$CN (HPLC quality), PCl$_3$ and HNEt$_2$, EM Science; HF, Matheson Gas Products.

Peptide synthesis

The peptides were prepared by standard solid-phase peptide methodology on an ABI 431A Peptide Synthesizer. Fmoc (ABI Standard Fmoc, Version 1.12) and Boc (ABI Standard Boc, Version 1.12) strategies were employed (16). Both utilized single couplings with four equivalents of the protected amino acid HOBT-activated ester formed by reaction with DCC. A typical cycle for the coupling of an individual amino acid by the Fmoc strategy was: (1) deprotection with 20% piperidine in NMP, 21 min; (2) washes with NMP,
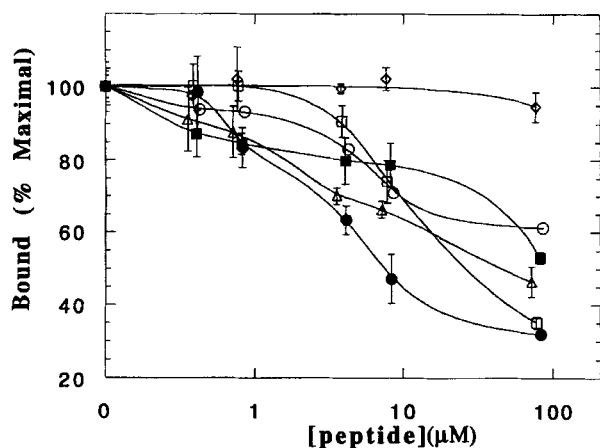

FIGURE 1

Inhibition of the binding of ^{35}S-labelled PLCγ SH2 fusion protein to phosphorylated EGFR by phosphorylated peptides. Values are expressed as percentage of the radioligand bound as a function of peptide concentration; **1**, ●; **3**, □; **5**, ■; **7**, ○; **9**, △; **11**, ◇.

9 min; (3) coupling of the HOBT-activated ester in NMP, 71 min; and (4) washes with NMP, 7 min. At the completion of the synthesis, the protected peptide resin was washed with DCM and dried at reduced pressure. A typical cycle for the coupling of an individual amino acid by the Boc strategy was: (1) deprotection with 25% TFA/DCM for 3 min; (2) deprotection with 50% TFA/DCM for 16 min; (3) washes with DCM, 3 min; (4) washes with 5% DIEA/DCM, 4 min; (5) washes with NMP, 5 min; (6) coupling of the HOBT-activated ester in NMP, 39 min; (7) addition of DMSO to make 15% DMSO/85% NMP, 16 min; (8) addition of 3.8 equiv. of DIEA, 5 min; (9) washes with NMP; (10) capping with 10% acetic anhydride, 5% DIEA in NMP, 9 min; and (11) washes with DCM, 4 min. After the last amino acid had been coupled, the N-terminal Boc group was removed, and the protected peptide resin was washed with DCM and dried at reduced pressure. The peptides synthesized by Fmoc strategy on HMP resins were obtained by treatment with TFA containing various scavengers. Time course experiments were performed on individual peptides to determine the optimal reaction time.

Peptide purification
The crude peptide was initially investigated by analytical HPLC to optimize the gradient used in its preparative purification. Then, approximately 100 mg of crude peptide were dissolved in 2.0 mL of 50% AcOH/H_2O, filtered through a 0.45 μm syringe filter, injected onto a 5.0 mL loop, and chromatographed on a Vydac 218TP1022 HPLC column (10 μm particle size, 300 Å pore size, 2.2 × 25 cm). A selected gradient of 0.1% TFA/CH_3CN (B) was used to elute the peptide from the column, which was originally in 100% 0.1% TFA/H_2O (A). For example, the gradient employed for 1 was: 0% B for 10 min, 0–8% B over 8 min, and 8–28% B over 120 min. A flow rate of 15 mL/min was maintained on a Varian 5000 liquid chromatograph and the absorbance of the eluant at 215 nm was recorded on a Hitachi D-2500 integrator. Individual fractions were collected and combined based upon analytical HPLC. The combined fractions were concentrated under reduced pressure, diluted with H_2O, and lyophilized to give the product.

Peptide analysis
The peptides were assayed for purity by analytical HPLC. One mg of the peptide was dissolved in 1 mL of H_2O, filtered through a 0.45 μm syringe filter, and chromatographed on a Vydac 218TP54 HPLC column (5 μm particle size, 300 Å pore size, 0.46 × 25 cm) using an appropriately selected gradient. A flow rate of 1.5 mL/min was maintained on a Varian 5500 liquid chromatograph and the absorbance of the eluant at 215 nm was recorded on a Hitachi D-2500 integrator.

The peptides were also assayed for purity by CZE using the same solutions prepared above on an ABI 270A analytical capillary electrophoresis system. A 72 cm × 50 μm ID capillary was used with a buffer of 20 mM Na citrate, pH 2.5, at a given voltage. The absorbance of the eluant at either 200 or 210 nm was recorded on a Hitachi D-2500 integrator.

Amino acid analyses were performed on an ABI 420A-H system utilizing automated hydrolysis and precolumn derivatization. Automated vapor-phase hydrolysis was performed at 180 °C in 6 N HCl for 75 min. HPLC analysis of the derivatized amino acids was performed on a 2.1 mm column using a gradient system of acetate buffered H_2O and CH_3CN with UV detection at 254 nm.

^{1}H NMR spectra were determined at 250 MHz on a Bruker AM250 spectrometer in D_2O or DMSO-d_6 with tetramethylsilane as an internal standard. For peptides 1–12, only the chemical shifts of the two doublets (d) due to the aromatic protons in the tyrosine moiety are reported. For peptides 13 and 15, the chemical shifts of the methylene protons adjacent to the phosphorus are reported in addition to the aromatic protons, as well as the methyl protons of 13.

The fast atom bombardment mass spectra (FAB-MS) were obtained on a VG 7070/E-HF mass spectrometer using a direct target probe, xenon target gas, and a thioglycerol matrix. Electrospray ionization mass spectra (EI-MS) were obtained from a solution of approximately 100 ng/μL of sample in MeOH/H_2O (50/50) with 3% AcOH. The solution was analyzed by direct infusion into a Vestec Electrospray interface to a Finnigan TSQ 70 mass spectrometer at an approximate flow rate of 5 μL/min. The observed $(M + 1)^+$ or $(M + 23)^+$ peak is reported with the calculated value for $(M + 1)^+$ or $(M + 23)^+$. In the cases of FAB-MS, this calculated value is the monoisotopic mass, whereas in the case of ES-MS, this calculated value is the average mass.

Biological assay
The details of the assay have been recently reported (10). Briefly, a bacterial glutathione–S-transferase fusion protein, containing both SH2 domains of PLCγ1, was labelled *in vivo* with ^{35}S. Phosphorylated EGFR was purified from EGF-treated 3T3 cells transfected with the human EGFR by precipitation with anti-EGFR antisera. The resulting immune complex was bound to protein A–Sepharose beads. Binding of the ^{35}S-labelled PLCγ1 SH2 fusion protein (25 nM) to the purified EGFR–beads complex (approximately 1 μg of receptor/assay) was allowed to proceed to equilibrium (20 min) in a total volume of 0.4 mL in the presence of the indicated concentrations of peptides. Bound ^{35}S fusion protein was separated from free by centrifugation, and radioactivity was determined by scintillation counting. Results are expressed in Fig. 1 as the means of triplicate determinations ± standard deviations, and were repeated in five separate experiments.

Asp-Ala-Asp-Glu-Tyr(PO₃H₂)-Leu-Ile-Pro-Gln-Gln-Gly-OH (1). The title compound was prepared by a Fmoc/t-Bu strategy by first loading the HMP resin (238 mg, 1.05 mmol/g, 0.25 mmol) with N^α-Fmoc-Gly symmetrical anhydride (0.5 mmol) using DMAP as catalyst. The peptide was elongated by the sequential coupling of the following amino acids (1 mmol) as their HOBT-activated esters (in order of addition): N^α-Fmoc-Gln, N^α-Fmoc-Gln, N^α-Fmoc-Pro, N^α-Fmoc-Ile, N^α-Fmoc-Leu, N^α-Fmoc-Tyr, N^α-Fmoc-Glu(O-t-Bu), N^α-Fmoc-Asp(O-t-Bu), N^α-Fmoc-Ala, N^α-Boc-Asp(O-t-Bu). The tyrosine was phosphorylated by adding one-half (350 mg, 0.12 mmol) of the lot (701 mg) to a rocking reaction vessel and washing sequentially with DMF (3 × 15 mL), DCM (3 × 15 mL), and anhydrous THF (3 × 15 mL). To the resin was added anhydrous THF (12 mL), followed by di-t-Bu-N,N-diethylphosphoramidite (17) (1.56 g, 6.25 mmol, 50 equiv.) and then tetrazole (1.32 g, 18.8 mmol, 150 equiv.). The suspension was rocked for 60 min, then drained and washed sequentially with anhydrous THF (3 × 15 mL), reagent grade (not dried) THF (10 × 15 mL), and DCM (7 × 15 mL). The resin was suspended in 12 mL of DCM and treated with t-Bu hydroperoxide (70% aqueous solution, 0.34 mL, 2.5 mmol, 20 equiv.) and rocked for 60 min. The suspension was drained, and the resin was washed sequentially with DCM (5 × 15 mL), DMF (5 × 15 mL) and DCM (5 × 15 mL), and dried under reduced pressure (359 mg).

The peptide was cleaved from the polystyrene resin and the side chains deblocked by stirring the peptide resin with a solution of TFA (9.5 mL) and H₂O (0.5 mL) for 2 h at room temperature. The spent resin was collected by filtration and discarded, and the filtrate was concentrated under reduced pressure, diluted with H₂O, and lyophilized (195 mg). Preparative HPLC of the crude peptide (150 mg) gave the product as a white solid (81 mg). HPLC: 0–15% B/45 min, 28.1 min (96.1%); CZE: + 30 kV, 200 nm, 44.4 min (96.5%); amino acid analysis: Asx (2) 2.14, Glx (3) 3.13, Gly (1) 1.22, Ala (1) 1.07, Pro (1) 0.99, Tyr (1) 0.97, Ile (1) 0.95, Leu (1) 1.00; ¹H-NMR (D₂O): δ 7.11 (d, J = 8.3 Hz, 2), 7.21 (d, J = 8.5 Hz, 2); ES-MS: $(M + H)^+$ = 1328.8 (calcd., 1329.3).

Asp-Ala-Asp-Glu-Tyr-Leu-Ile-Pro-Gln-Gln-Gly-OH (2). The title compound was prepared from the same intermediate resin as was 1. The peptide was cleaved by treatment of the nonphosphorylated peptide resin (227 mg) as in 1 (122 mg). Preparative HPLC of the crude peptide (105 mg) gave the product as a white solid (60 mg). HPLC: 0–15% B/45 min, 33.6 min (96.0%); CZE: + 20 kV, 200 nm, 32.4 min (83.3%); amino acid analysis: Asx (2) 2.22, Glx (3) 2.26, Gly (1) 1.47, Ala (1) 1.28, Pro (1) 1.03, Tyr (1) 0.56, Ile (1) 1.04, Leu (1), 1.00; ¹H-NMR (D₂O): δ 6.81 (d, J = 8.3 Hz, 2), 7.12 (d, J = 8.3 Hz, 2); ES-MS $(M + H)^+$ = 1248.2 (calcd., 1249.3).

Phe-Leu-Pro-Val-Pro-Glu-Tyr(PO₃H₂)-Ile-Asn-Gln-Ser-Val-OH (3). The peptide resin was prepared by a Fmoc/t-Bu strategy by first loading the HMP resin (294 mg, 0.85 mmol/g, 0.25 mmol) with N^α-Fmoc-Val symmetrical anhydride (0.5 mmol) using DMAP as catalyst. The peptide was elongated by the sequential coupling of the following amino acids (1 mmol) as their HOBT-activated esters (in order of addition): N^α-Fmoc-Ser(t-Bu), N^α-Fmoc-Gln, N^α-Fmoc-Asn, N^α-Fmoc-Ile, N^α-Fmoc-Tyr, N^α-Fmoc-Glu(O-t-Bu), N^α-Fmoc-Pro, N^α-Fmoc-Val, N^α-Fmoc-Pro, N^α-Fmoc-Leu, N^α-Boc-Phe. One half (363 mg) of the lot (727 mg) was phosphorylated as in 1 (379 mg). The peptide was cleaved as in 1, but for 7 h (193 mg). Preparative HPLC of the crude peptide (97 mg) gave the product as a white solid (52 mg). HPLC: 10–50% B/45 min, 17.9 min (98.5%); CZE: + 30 kV, 200 nm, 26.0 min (100%); amino acid analysis: Asx (1) 1.14, Glx (2) 2.15, Ser (1) 0.97, Pro (2) 1.94, Tyr (1) 0.89, Val (2) 1.98, Ile (1) 0.95, Leu (1) 1.00, Phe (1) 0.98; ¹H-NMR (D₂O): δ 7.11 (d, J = 8.3 Hz, 2), 7.19 (d, J = 8.5 Hz, 2); ES-MS: $(M + H)^+$ = 1485.1 (calcd., 1486.6).

Phe-Leu-Pro-Val-Pro-Glu-Tyr-Ile-Asn-Gln-Ser-Val-OH (4). The title compound was prepared from the same intermediate resin as was 3. The peptide was cleaved by treatment of the nonphosphorylated peptide resin (155 mg) as in 3 (71 mg). Preparative HPLC gave the product as a white solid (45 mg). HPLC: 10–50% B/45 min, 21.4 min (97.6%); CZE: + 30 kV, 200 nm, 12.4 min (99.5%); amino acid analysis: Asx (1) 1.00, Glx (2) 1.79, Ser (1) 0.87, Pro (2) 1.54, Tyr (1) 0.74, Val (2) 1.62, Ile (1) 0.99, Leu (1) 1.05, Phe (1) 0.84; ¹H-NMR (D₂O): δ 6.81 (d, J = 8.4 Hz, 2), 7.10 (d, J = 8.5 Hz, 2); ES-MS: $(M + H)^+$ = 1405.9 (calcd., 1406.6).

Asp-Asn-Pro-Asp-Tyr(PO₃H₂)-Gln-Gln-Asp-Phe-Phe-OH (5). The peptide resin was prepared by a Fmoc/t-Bu strategy by first loading the HMP resin (238 mg, 1.05 mmol/g, 0.25 mmol) with N^α-Fmoc-Phe symmetrical anhydride (0.5 mmol) using DMAP as catalyst. The peptide was elongated by the sequential coupling of the following amino acids (1 mmol) as their HOBT-activated esters (in order of addition): N^α-Fmoc-Phe, N^α-Fmoc-Asp(Ot-Bu), N^α-Fmoc-Gln, N^α-Fmoc-Gln, N^α-Fmoc-Tyr, N^α-Fmoc-Asp(Ot-Bu), N^α-Fmoc-Pro, N^α-Fmoc-Asn, N^α-Boc-Asp(O-t-Bu). One-half (330 mg) of the lot (660 mg) was phosphorylated as in 1 (354 mg). The peptide was cleaved as in 1 but for 18 h (155 mg). Preparative HPLC gave the product as a white solid (45 mg). HPLC: 10–50% B/22 min, 11.3 min (99.2%); CZE: + 20 kV, 210 nm, no peak (30 min); amino acid analysis: Asx (4) 3.73, Glx (2) 2.40, Pro (1) 1.00, Tyr (1) 0.90, Phe (2) 1.83; ¹H-NMR (DMSO): δ 7.05 (d, J = 8.7 Hz, 2), 7.13 (d, J = 9.7 Hz, 2); FAB-MS: $(M + H)^+$ = 1369.3 (calcd., 1368.5).

Asp-Asn-Pro-Asp-Tyr-Gln-Gln-Asp-Phe-Phe-OH(6). The title compound was prepared by a Boc/Bzl strategy using Boc-Phe-PAM resin (860 mg, 0.58 mmol/g, 0.5 mmol). The peptide was elongated by the sequential coupling of the following amino acids (2 mmol) as their HOBT-activated esters (in order of addition): N^α-Boc-Phe, N^α-Boc-Asp(O-Bzl), N^α-Boc-Gln, N^α-Boc-Gln, N^α-Boc-Tyr(2-bromo-benzyloxycarbonyl) (BrZ), N^α-Boc-Asp(*O*-Bzl), N^α-Boc-Pro, N^α-Boc-Asn, N^α-Boc-Asp(*O*-Bzl). Five hundred milligrams of the total lot (1.64 g) were treated as in **8** to give crude material (134 mg). Preparative HPLC gave the product as a white solid (71 mg). HPLC: 10–50% B/22 min, 13.0 min (99.0%); CZE: +20 kV, 210 nm, 26.7 min (98.3%); amino acid analysis: Asx (4) 4.14, Glx (2) 2.06, Pro (1) 0.94, Tyr (1) 0.92, Phe (2) 2.00; ^{1}H-NMR (DMSO): δ 6.62 (d, $J = 8.4$ Hz, 2), 6.98 (d, $J = 8.3$ Hz, 2); FAB-MS: $(M + H)^+ = 1288.9$ (calcd., 1288.5).

Ala-Glu-Tyr(PO₃H₂)-Leu-Arg-Val-Ala-Pro-Gln-Ser-OH (7). The protected peptide resin was prepared by a Fmoc/t-Bu strategy by first loading the HMP resin (238 mg, 1.05 mmol/g, 0.25 mmol) with N^α-Fmoc-Ser(t-Bu) symmetrical anhydride (0.5 mmol) using DMAP as catalyst. The peptide was elongated by the sequential coupling of the following amino acids (1 mmol) as their HOBT-activated esters (in order of addition): N^α-Fmoc-Gln, N^α-Fmoc-Pro, N^α-Fmoc-Ala, N^α-Fmoc-Val, N^α-Fmoc-Arg (N^G-2,2,5,7,8-pentamethylchroman-6-sulfonyl)(Pmc), N^α-Fmoc-Leu, N^α-Fmoc-Tyr, N^α-Fmoc-Glu(*O*-t-Bu), N^α-Boc-Ala. One half (312 mg) of the lot (624 mg) was phosphorylated as in **1** (312 mg). The peptide was cleaved as in **9**, but for 4.5 h (141 mg). Preparative HPLC gave the product as a white solid (95 mg). HPLC: 10–50% B/22 min, 8.7 min (99.0%); CZE: +20 kV, 210 nm, 21.6 min (99.2%); amino acid analysis: Glx (2) 2.30, Ser (1) 1.06, Arg (1) 1.00, Ala (2) 2.28, Pro (1) 1.08, Tyr (1) 1.07, Val (1) 1.16, Leu (1) 1.19; ^{1}H-NMR (D₂O): δ 6.99 (d, $J = 8.7$ Hz, 2), 7.07 (d, $J = 8.6$ Hz, 2); FAB-MS: $(M + H)^+ = 1213.9$ (calcd., 1213.5).

Ala-Glu-Tyr-Leu-Arg-Val-Ala-Pro-Gln-Ser-OH (8). The title compound was prepared by a Boc/Bzl strategy using Boc-Ser(Bzl)-PAM resin (690 mg, 0.72 mmol/g, 0.5 mmol). The peptide was elongated by the sequential coupling of the following amino acids (2 mmol) as their HOBT-activated esters (in order of addition): N^α-Boc-Gln, N^α-Boc-Pro, N^α-Boc-Ala, N^α-Boc-Val, N^α-Boc-Arg (N^G-tosyl), N^α-Boc-Leu, N^α-Boc-Tyr(BrZ), N^α-Boc-Glu(O-Bzl), N^α-Boc-Ala. Five hundred milligrams of the total lot (1.484 g) were treated with anhydrous HF (10 mL) and anisole (1 mL) (1 h, 0 °C). The HF was removed by a stream of N₂. The peptide and resin were triturated with ether and collected by filtration. The peptide was extracted with 50% AcOH/H₂O (4 × 25 mL), and the extracts were concentrated under reduced pressure, diluted with H₂O, and lyophilized

(203 mg). Preparative HPLC of the crude peptide (186 mg) gave the product as a white solid (130 mg). HPLC: 10–50% B/22 min, 9.7 min (98.9%); CZE: +20 kV, 210 nm, 13.4 min (98.5%); amino acid analysis: Glx (2) 1.97, Ser (1), 1.03, Arg (1) 0.98, Ala (2) 2.02, Pro (1) 0.90, Tyr (1) 0.90, Val (1) 0.99, Leu (1) 1.00; ^{1}H-NMR (D₂O): δ 6.70 (d, $J = 8.5$ Hz, 2); 7.01 (d, $J = 8.5$ Hz, 2); FAB-MS: $(M + H)^+ = 1133.5$ (calcd., 1133.6).

Ala-Glu-Tyr(PO₃H₂)-Leu-Arg-Val-Ala-OH (9). The peptide resin was prepared by a Fmoc/t-Bu strategy by first loading the HMP resin (294 mg, 0.85 mmol/g, 0.25 mmol) with N^α-Fmoc-Ala symmetrical anhydride (0.5 mmol) using DMAP as catalyst. The peptide was elongated by the sequential coupling of the following amino acids (1 mmol) as their HOBT-activated esters (in order of addition): N^α-Fmoc-Val, N^α-Fmoc-Arg(Pmc), N^α-Fmoc-Leu, N^α-Fmoc-Tyr, N^α-Fmoc-Glu(*O*-t-Bu), and N^α-Boc-Ala. One half (318 mg) of the lot (635 mg) was phosphorylated as in **1** (329 mg). This was stirred with a solution of TFA (10 mL), EDT (0.25 mL), thioanisole (0.5 mL), phenol (0.75 g) and H₂O (0.5 mL) for 3.5 h. The spent resin was collected by filtration and discarded. The filtrate was concentrated under reduced pressure to give a syrup which was diluted with ether, affording a solid. This was collected, dissolved in H₂O, and lyophilized (119 mg). Preparative HPLC gave the product as a white solid (68 mg). HPLC: 0–15% B/45 min, 15.3 min (95.8%), 22.0 min (**10**, 4.2%); CZE: +20 kV, 200 nm, 20.3 min (97.6%); amino acid analysis: Glx (1) 1.17, Arg (1) 0.92, Ala (2) 2.17, Tyr (1) 0.93, Val (1) 0.99, Leu (1) 1.00; ^{1}H-NMR (D₂O): δ 7.09 (d, $J = 8.6$ Hz, 2) 7.18 (d, $J = 8.6$ Hz, 2); ES-MS: $(M + H)^+ = 901.3$ (calcd., 901.9).

Ala-Glu-Tyr-Leu-Arg-Val-Ala-OH (10). The title compound was prepared from the same intermediate resin as was **9**. The peptide was cleaved by treatment of the nonphosphorylated peptide resin (245 mg) as in **9**, but for 2 h (102 mg). Preparative HPLC gave the product as a white solid (74 mg). HPLC: 0–15% B/45 min, 21.3 min (100%); CZE: +20 kV, 200 nm, 11.8 min (99.5%); amino acid analysis: Glx (1) 1.11, Arg (1) 0.92, Ala (2) 1.81, Tyr (1) 0.90, Val (1) 0.92, Leu (1) 1.00; ^{1}H-NMR (D₂O): δ 6.80 (d, $J = 8.3$ Hz, 2) 7.11 (d, $J = 8.3$ Hz, 2); ES-MS: $(M + H)^+ = 821.0$ (calcd., 821.9).

Tyr(PO₃H₂)-Leu-Arg-Val-Ala-Pro-Gln-Ser-OH(11). The peptide resin was prepared by a Fmoc/t-Bu strategy by first loading the HMP resin (294 mg, 0.85 mmol/g, 0.25 mmol) with N^α-Fmoc-Ser(t-Bu) symmetrical anhydride (0.5 mmol) using DMAP as catalyst. The protected peptide was elongated by the sequential coupling of the following amino acids (1 mmol) as their HOBT-activated esters (in order of addition): N^α-Fmoc-Gln,

N^α-Fmoc-Pro, N^α-Fmoc-Ala, N^α-Fmoc-Val, N^α-Fmoc-Arg(Pmc), N^α-Fmoc-Leu, N^α-Boc-Tyr. One half (294 mg) of the lot (587 mg) was phosphorylated as in 1 (308 mg). The peptide was cleaved as in 9 (128 mg). Preparative HPLC gave the product as a white solid (66 mg). HPLC: 10–20% B/45 min, 20.5 min (98.5%); CZE: + 20 kV, 200 nm, 24.0 min (99.8%); amino acid analysis: Glx (1) 0.99, Ser (1) 0.93, Arg (1) 0.94, Ala (1) 1.08, Pro (1) 1.00, Tyr (1) 0.95, Leu (1) 1.10, Val (1) 1.02; ^{1}H-NMR (D$_2$O): δ 7.02 (d, J = 8.9 Hz, 2) 7.07 (d, J = 8.9 Hz, 2); ES-MS: $(M + H)^+$ = 1014.1 (calcd., 1014.1).

Tyr-Leu-Arg-Val-Ala-Pro-Gln-Ser-OH (12). The title compound was prepared form the same intermediate resin as was 11. The peptide was cleaved by treatment of the nonphosphorylated peptide resin (210 mg) as in 10) (95 mg). Preparative HPLC gave the product as a white solid (73 mg). HPLC: 10–20% B/45 min, 23.0 min (98.8%); CZE: + 20 kV, 200 nm, 13.8 min (99.4%); amino acid analysis: Glx (1) 1.04, Ser (1) 0.99, Arg (1) 1.04, Ala (1) 1.09, Pro (1) 1.00, Tyr (1) 0.95, Val (1) 1.09, Leu (1) 1.17; ^{1}H-NMR (D$_2$O): δ 6.73 (d, J = 8.5 Hz, 2) 7.00 (d, J = 8.5 Hz, 2); ES-MS: $(M + H)^+$ = 933.4 (calcd., 934.1).

Asp-Ala-Asp-Glu-Phe[4-CH$_2$PO(OCH$_3$)$_2$]-Leu-Ile-Pro-Gln-Gln-Gly-OH (13) and Acetyl-Leu-Ile-Pro-Gln-Gln-Gly-OH (14). The title compound 13 was prepared by a Boc/Bzl strategy using Boc-Gly-PAM resin (707 mg, 0.70 mmol/g, 0.5 mmol). The peptide was elongated by the sequential coupling of the following amino acids (2 mmol) as their HOBT-activated esters (in order of addition): N^α-Boc-Gln, N^α-Boc-Gln, N^α-Boc-Pro, N^α-Boc-Ile, N^α-Boc-Leu, N^α-Boc-Phe[4-CH$_2$PO(OCH$_3$)$_2$] (14), N^α-Boc-Glu(O-Bzl), N^α-Boc-Asp(O-cyclohexyl), N^α-Boc-Ala, N^α-Boc-Asp(O-cyclohexyl). Six hundred milligrams of the total lot (1.222 g) were treated as in 8 except p-cresol was substituted for anisole. Preparative HPLC of the crude peptide (108 mg of the total 225 mg) gave 13 as a white solid (58 mg). HPLC: 10–40% B/45 min, 23.6 min (96.4%); CZE: + 20 kV, 210 nm, 33.5 min (90.3%); amino acid analysis: Asx (2) 0.96, Glx (3) 2.11, Gly (1) 0.87, Ala (1) 1.00, Pro (1) 0.83, Ile (1) 0.96, Leu (1) 1.00; dimethylphosphonomethylphenylalanine (1) not determined; ^{1}H-NMR (D$_2$O): δ 3.34 (d, J = 21.4 Hz, 2, CH$_2$P), 3.72 (d, J = 10.9 Hz, 6, OMe), 7.25 (multiplet, 4); FAB-MS: $(M + Na)^+$ = 1376.2 (calcd., 1376.6).

Also isolated in the preparative HPLC of the crude peptide was 14 as a white solid (28 mg). HPLC: 10–40% B/45 min, 19.7 min (97.7%); CZE: + 25 kV, 210 nm, 37.6 min (100%); amino acid analysis: Glx (2) 2.25, Gly (1) 0.58, Pro (1) 0.75, Ile (1) 1.00, Leu (1) 0.90; FAB-MS: $(M + 23)^+$ = 719.3 (calcd., 719.4).

Asp-Ala-Asp-Glu-Phe (4-CH$_2$PO$_3$H$_2$)-Leu-Ile-Pro-Gln-Gln-Gly-OH (15). The title compound was prepared by treatment of 13 (20 mg) with a solution of TFMSA/TFA/DMS/m-cresol (0.2 mL/1 mL/0.6 mL/0.2 mL) at 0 °C for 2 h. The solution was diluted with ether to afford a solid, which was collected by filtration, dissolved in H$_2$O, and lyophilized to give a hygroscopic solid. Preparative HPLC of the crude peptide gave the product as a white solid (12 mg). HPLC: 10–25% B/45 min, 29.0 min (96.6%); CZE: + 25 kV, 210 nm, 39.1 min (100%); amino acid analysis: Asx (2) 1.70, Glx (3) 2.38, Gly (1) 1.05, Ala (1) 1.00, Pro (1) 1.39, Ile (1) 1.09, Leu (1) 0.91, Pmp (1) 1.31 [elutes with the approximate retention time of His (18)]; ^{1}H-NMR (D$_2$O): δ 3.0 (d, J = 21 Hz, 2, CH$_2$P), 7.2 (multiplet, 4); FAB-MS: $(M + H)^+$ = 1326.6 (calcd., 1326.6). Also isolated in the preparative HPLC was a by-product as a hygroscopic white solid (3 mg). HPLC: 10–25% B/45 min, 30.9 min (88.9%). FAB-MS: $(M + H)^+$ = 1308.6 [calcd. for $(M + H)^+$ of 15 minus 18(H$_2$O), 1308.6].

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