SH2 domains prevent tyrosine dephosphorylation of the EGF receptor: identification of Tyr992 as the high-affinity binding site for SH2 domains of phospholipase C γ

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Communicated by J.Schlessinger

Several cytoplasmic tyrosine kinases contain a conserved, non-catalytic stretch of ~100 amino acids called the *src* homology 2 (SH2) domain, and a region of ~50 amino acids called the SH3 domain. SH2/SH3 domains are also found in several other proteins, including phospholipase C- γ (PLC γ). Recent studies indicate that SH2 domains promote association between autophosphorylated growth factor receptors such as the epidermal growth factor (EGF) receptor and signal transducing molecules such as PLC γ . Because SH2 domains bind specifically to protein sequences containing phosphotyrosine, we examined their capacity to prevent tyrosine dephosphorylation of the EGF and other receptors with tyrosine kinase activity. For this purpose, various SH2/SH3 constructs of PLC γ were expressed in *Escherichia coli* as glutathione-S-transferase fusion proteins. Our results show that purified SH2 domains of PLC γ are able to prevent tyrosine dephosphorylation of the EGF receptor and other receptors with tyrosine activity. The inhibition of tyrosine dephosphorylation paralleled the capacity of various SH2-containing constructs to bind to the EGF receptor, suggesting that the tyrosine phosphatase and the SH2 domain compete for the same tyrosine phosphorylation sites in the carboxy-terminal tail of the EGF receptor. Analysis of the phosphorylation sites protected from dephosphorylation by PLC γ -SH2 revealed substantial inhibition of dephosphorylation of Tyr992 at 1 μ M SH2. This indicates that Tyr992 and its flanking sequence is the high-affinity binding site for SH2 domains of PLC γ . Higher concentrations of PLC γ -SH2 also led to the protection from dephosphorylation of Tyr1068 (≥ 1 μ M), Tyr1173 (≥ 6 μ M) and Tyr1086 (≥ 100 μ M). These results provide further support for the central regulatory role of SH2 domains in signal transduction pathways, and reveal a hierarchy in the interactions between tyrosine phosphorylation sites of the EGF receptor and PLC γ -SH2.

Key words: receptors/signal transduction/tyrosine kinases/tyrosine phosphatases

Introduction

Tyrosine phosphorylation of cellular target proteins plays a crucial role in the control of cell growth, differentiation and oncogenesis (reviewed in Hanks *et al.*, 1988; Williams, 1989; Carpenter and Cohen, 1990; Ullrich and Schlessinger, 1990). The net cellular level of tyrosine phosphorylation is maintained by enzymes which catalyze the incorporation (i.e. kinases) or removal (i.e. phosphatases) of phosphate from tyrosine residues. Tyrosine kinases comprise either ligand-dependent growth factor receptors such as epidermal growth factors (EGF) or platelet-derived growth factor (PDGF) receptors (EGFR, PDGFR) (reviewed in Williams, 1989; Carpenter and Cohen, 1990; Ullrich and Schlessinger, 1990) or cytoplasmic kinases such as pp60^{c-src} and p56^{lck} (Hanks *et al.*, 1988; Veillette *et al.*, 1989; Eisenman and Bolen, 1990). Recent evidence indicates that some cytoplasmic tyrosine kinases are also activated by interactions with surface receptors (Veillette *et al.*, 1989a). Tyrosine phosphatases also comprise cytoplasmic and receptor-type enzymes [reviewed in Fischer *et al.* (1991) and Saito and Streuli (1991)]. The various factors and cellular components which regulate tyrosine dephosphorylation are poorly understood. It is likely, however, that tyrosine dephosphorylation is also a highly regulated cellular process.

The cellular phosphotyrosine content was shown to be regulated not only by proteins which possess intrinsic protein tyrosine kinase or phosphatase activities, but also by an oncogenic protein called *v-crck*, which consists essentially of an SH2 and SH3 domain fused to viral *gag* protein (Mayer *et al.*, 1988; Matsuda *et al.*, 1990). Moreover, the *v-crck* protein was shown to be complexed to several tyrosine-phosphorylated proteins in *v-crck* transformed cells (Matsuda *et al.*, 1990, 1991; Mayer and Hanafusa, 1990). It has been proposed that *v-crck* induces transformation by regulating the enzymatic activities of tyrosine kinases and/or tyrosine phosphatases (Matsuda *et al.*, 1991). *src* homology 2 (SH2) and SH3 domains were also found in phospholipase C- γ (PLC γ) (Stahl *et al.*, 1988; Suh *et al.*, 1988; Rhee *et al.*, 1989), GTPase activating protein of *ras* (GAP) (Trahey *et al.*, 1988; Vogel *et al.*, 1988), *nck* (Lehmann *et al.*, 1990), the proto-oncogene *vav* (Katzav *et al.*, 1989), tensin (Davis *et al.*, 1991) and the PI3 kinase-associated p85 (GRB1) (Escobedo *et al.*, 1991a; Otsu *et al.*, 1991; Skolnik *et al.*, 1991). Both PLC γ and GAP have an important function in signal transduction pathways. Activation of PLC γ leads to the generation of diacylglycerol and inositol triphosphate, which in turn activate protein kinase C and mobilize intracellular calcium. GAP enhances the GTPase activity of *ras* and is therefore likely to be involved in the regulation of *ras* action.

Several laboratories have shown that activation of either EGF or PDGF receptors leads to tyrosine phosphorylation

of and complex formation with either GAP or PLC γ (Margolis *et al.*, 1989a, 1990a,b; Meisenhender *et al.*, 1989; Molloy *et al.*, 1989; Wahl *et al.*, 1989; Ellis *et al.*, 1990; Kazlauskas *et al.*, 1990; Kaplan *et al.*, 1990a). Moreover, tyrosine phosphorylation has been shown to be essential for activation of PLC γ by growth factors (Nishibe *et al.*, 1990; Kim *et al.*, 1991). The physical association between activated EGFR and GAP or PLC γ is mediated by previously unidentified tyrosine-autophosphorylated site(s) located within the carboxy-terminal tail of the EGFR and the SH2 domains of signaling proteins (Anderson *et al.*, 1990; Margolis *et al.*, 1990b; Moran *et al.*, 1990; Skolnik *et al.*, 1991). As SH2 domains of GAP or PLC γ interact with the tyrosine-phosphorylated tail of the EGFR, we have reasoned that SH2 domains may compete with tyrosine-specific phosphatases for the same region and thus prevent tyrosine dephosphorylation of EGFR. In accordance, we demonstrate in this report that purified recombinant SH2 domains of either PLC γ or GAP are able to prevent dephosphorylation of the EGFR and other receptors with tyrosine kinase activity. Moreover, utilizing the ability of the SH2 domains to prevent tyrosine dephosphorylation, we have identified the tyrosine phosphorylation sites which bind the SH2 domains of PLC γ . Using this approach, we have now identified Tyr992 as the high-affinity binding site of the EGFR towards PLC γ -SH2.

Results

It is well established that following EGF-induced tyrosine autophosphorylation, the EGFR is rapidly dephosphorylated by a vanadate-sensitive tyrosine phosphatase(s) (Swarup

et al., 1982; Butler *et al.*, 1989); a finding also confirmed by our study. Almost complete dephosphorylation of the EGFR was obtained after a 10 min incubation with an A431 cell lysate at 37°C, and a much slower rate of dephosphorylation was achieved at 4°C (data not shown).

To test whether the SH2/SH3 domains of PLC γ can inhibit EGFR dephosphorylation, we have constructed a fusion protein of glutathione-S-transferase (GST)-SH2-SH2-SH3 (construct I) of the human PLC γ , which was expressed in the pGEX-3X bacterial expression system (Figure 1). The addition of this fusion protein, but not GST alone, to the reaction mixture containing A431 cell lysate virtually prevented dephosphorylation of the EGFR (Figure 2A). Similar inhibition of phosphatase activity was obtained with 200 μ M vanadate (Figure 2A). The extent of inhibition of EGFR dephosphorylation by construct I was dose dependent (Figure 3), with an estimated IC₅₀ value of $\sim 5-10$ μ M. In order to further delineate the region(s) involved in the prevention of tyrosine dephosphorylation of EGFR, we generated additional recombinant fragments of PLC γ (Figure 1) and compared their capacity to either bind to tyrosine-phosphorylated EGFR or to prevent tyrosine dephosphorylation. Figure 2A and B shows that the pattern of both responses is virtually identical; namely, the capacity to bind to EGF-stimulated tyrosine-autophosphorylated EGFR parallels the ability of the various recombinant fragments to prevent tyrosine dephosphorylation of EGFR molecules. Interestingly, the SH3 domain (construct III in Figure 1) and the region containing the two phosphorylation sites alone (construct VI in Figure 1) neither bound to EGFR nor prevented tyrosine dephosphorylation of the receptor (Figure 2A and B). However, all the SH2-containing

Fig. 1. Generation of various SH2/SH3-containing PLC γ constructs. (A) Schematic representation of the various SH2/SH3-containing constructs of human PLC γ (Burgess *et al.*, 1990) and several constructs containing parts of this region. Construct I (wt) encompassing nucleotides 1697–2633, construct II (2SH2-P) encompassing nucleotides 1697–2468, construct III (P-SH3) encompassing nucleotides 2358–2633, construct IV (SH2-P-SH3) encompassing nucleotides 2031–2633, construct V (2SH2) encompassing nucleotides 1697–2366, construct VI (P) encompassing nucleotides 2358–2468 and construct VII (C-SH2) encompassing nucleotides 2031–2366. (B) Recombinant fusion proteins produced by each of the DNA constructs shown in (A) using the pGEX-3X expression system. Oligonucleotides based on the boundaries of the SH2/SH3 domains of human PLC γ cDNA were used to amplify the desired stretches of DNA by PCR. The DNA inserts were ligated in pGEX-3X bacterial expression plasmid and the recombinant plasmid introduced into *E. coli*. Expression of the GST fusion proteins was induced with 1 μ M IPTG and the fusion proteins were isolated from bacterial lysates utilizing affinity chromatography with glutathione-agarose beads, followed by elution with glutathione.

domains (constructs I, II, IV, V and VII) were able to bind to EGFR and to prevent tyrosine dephosphorylation of the receptor molecule (Figure 2A and B). Parallel control immunoblotting experiments with anti-EGFR antibodies confirmed that recombinant SH2 domains prevent receptor dephosphorylation and not receptor degradation. In these experiments the phosphorylated EGFR was incubated with lysates from A431 cells in the presence of SH2-lacking constructs (III and IV) or an SH2-containing construct (II). The receptor was subsequently subjected to immunoblotting analysis with either anti-peptide antiserum against a synthetic peptide from the carboxy-terminal tail of EGFR (anti-C) or with anti-phosphotyrosine antibodies (anti-PTyr). Immunoblotting with anti-C antibodies revealed the presence of a similar amount of EGFR in all the samples, regardless of the treatment (Figure 4A). These data are also supported by the finding that following dephosphorylation in the presence of SH2-lacking constructs (III and VI), rephosphorylation of the EGFR was achieved in all the treatment groups, regardless of the type of construct present during the previous dephosphorylation step (Figure 4B).

To determine whether the capacity of the SH2 domain(s) to prevent tyrosine dephosphorylation is a more general phenomenon, we tested the ability of PLC γ -SH2 to block dephosphorylation of other receptor tyrosine kinases, namely the PDGFR and the fibroblast growth factor receptor

(FGFR). In parallel, we also tested the ability of a similar GST fusion protein containing the SH2 domain of GAP to inhibit EGFR dephosphorylation. Our results (Figure 5A) show that dephosphorylation of autophosphorylated PDGFR with phosphatase from HER14 cell lysate was prevented by 100 μ M PLC γ -SH2 (construct II). Similarly, dephosphorylation of the FGFR by A431 cell lysate, or lysate from NIH 3T3 cells overexpressing the FGFR, was also inhibited by PLC γ -SH2 (constructs I and II) (data not shown). Moreover, 20–100 μ M of recombinant GST fusion protein of GAP-SH2-SH3-SH2 domains effectively inhibited tyrosine dephosphorylation of the EGFR (Figure 5B). Interestingly, the N-terminal SH2 domain of GAP at a concentration of 20 μ M was sufficient to confer this inhibition (not shown).

The tyrosine phosphatase(s) in either A431 or HER14 lysates, which are responsible for dephosphorylation of the EGFR, have not been identified. Hence, we have tested whether a purified known protein tyrosine phosphatase (PTP) could dephosphorylate the EGFR, and whether this dephosphorylation is prevented by SH2 domains. For these studies, we chose the human receptor type phosphatase- α (RPTP α) which was cloned in our laboratory (Kaplan *et al.*, 1990b; Sap *et al.*, 1990). RPTP α was expressed in insect cells following infection with recombinant baculovirus, and subsequently purified and characterized (Daum *et al.*, 1991). Figure 6 shows that purified RPTP α , assayed at optimal con-

Fig. 2. Recombinant SH2-containing PLC γ constructs inhibit dephosphorylation of EGFR. (A) Inhibition of dephosphorylation of the EGFR by the indicated constructs shown in Figure 1A or by sodium orthovanadate (VO $_4$). The EGFR from A431 cells was immunoprecipitated and autophosphorylated in the presence of 10 μ Ci [γ - 32 P]ATP, 4.5 μ M unlabeled ATP and 13 mM MnCl $_2$ as described in Materials and methods. The tyrosine-autophosphorylated EGFR was washed thoroughly and incubated in the presence of 100 μ l HEPES solution (control), 80 μ l A431 cell lysate plus 20 μ l HEPES solution, either alone or containing 100 μ M of constructs I–VII, GST alone or 200 μ M sodium orthovanadate, for 30 min at 37°C. The reaction was stopped with sample buffer and proteins were separated on 10% SDS–PAGE and analyzed by autoradiography (exposure time ~5 min). Similar inhibition of dephosphorylation was also observed with 20 μ M of SH2-containing constructs. (B) Immunoblots showing the association of constructs I–VII with EGF-stimulated (+) or unstimulated (–) EGFRs. HER14 cells were starved in 1% calf serum overnight and incubated with either PBS (control) or 40 nM EGF for 2 min at 37°C. Cells were lysed in lysis buffer containing protease and phosphatase inhibitors, as described in Materials and methods. Approximately 5 μ g of the indicated constructs bound to glutathione–agarose beads were incubated for 2 h with 800 μ l of the above lysates. After washing the samples were analyzed by SDS–PAGE (8%), transferred to nitrocellulose filters and subjected to immunoblotting analysis with antibodies directed against a synthetic peptide from the carboxy-terminal tail of EGFR (anti-C). Differences in the intensity of the bands reflect variations in the amount of protein bound to the glutathione–agarose beads (exposure time 6 h at –80°C).

Fig. 3. Dose response of inhibition of EGFR dephosphorylation by an SH2-containing PLC γ construct I. Increasing concentrations of construct I were incubated with tyrosine-autophosphorylated EGFR in the presence of A431 lysates as in Figure 2 (A). Quantification of the radioactive content of tyrosine-phosphorylated EGFR as a function of SH2 concentration (B). Solid and empty squares represent data from two separate experiments (exposure time ~ 5 min at 24°C).

Fig. 4. SH2 domains of PLC γ inhibit EGFR dephosphorylation and not receptor degradation. (A) Immunoblots of the EGFR analyzed with either antibodies directed against a synthetic peptide from the C terminus of EGFR (anti-C) or anti-phosphotyrosine (anti-PTyr) antibodies following dephosphorylation with A431 lysates in the presence of constructs II, III or VI. The experiment was carried out as in Figure 2A, except that autophosphorylation of EGFR was carried out in the absence of [γ - ^{32}P]ATP and in the presence of $30 \mu\text{M}$ unlabeled ATP and 13 mM MnCl_2 . Following dephosphorylation in the presence of A431 lysates without or with $50 \mu\text{M}$ of the indicated constructs (II, III and VI), the proteins were separated on 10% SDS-PAGE gels and then analyzed by immunoblotting with either anti-EGFR (anti-C) or anti-PTyr antibodies (exposure time 1 h at -80°C). (B) EGFR dephosphorylation by A431 cell lysates in the presence of $50 \mu\text{M}$ of constructs II, III and VI, followed by receptor rephosphorylation. Following dephosphorylation, one set of samples was analyzed by SDS-PAGE directly and the duplicate set was washed three times to remove lysate and subjected to rephosphorylation of the EGFR [with $10 \mu\text{Ci}$ [γ - ^{32}P]ATP, $4.5 \mu\text{M}$ unlabeled ATP and 13 mM MnCl_2 , see Honegger *et al.* (1988)], followed by SDS-PAGE analysis and autoradiography (exposure time ~ 30 min at 24°C).

Fig. 5. Inhibition of PDGFR dephosphorylation by PLC γ -SH2, and inhibition of EGFR dephosphorylation by GAP-SH2. (A) Inhibition of dephosphorylation of PDGFR by PLC γ -SH2 (construct II). The PDGFR was immunoprecipitated from serum-starved HER14 cells, stimulated with 10 ng/ml PDGF and autophosphorylated as described in Materials and methods. The PDGFR was dephosphorylated in the presence of HER14 cell lysate containing $100 \mu\text{M}$ GST, $100 \mu\text{M}$ construct II, or in the presence of $200 \mu\text{M}$ sodium orthovanadate (VO_4). Control samples were incubated in HEPES solution instead of cell lysate (exposure time ~ 1 h at 24°C). (B) Inhibition of dephosphorylation of EGFR by GAP-SH2-SH3-SH2. The EGFR was immunoprecipitated from A431 cells as described in the legend to Figure 2A, subjected to autophosphorylation reaction and treated with lysates from A431 cells in the absence or presence of $100 \mu\text{M}$ of the SH2-SH3-SH2 construct of GAP expressed in pGEX-3X (encompassing nucleotides 539–1325; Trahely *et al.*, 1988). Similar results were obtained when $20 \mu\text{M}$ of GAP protein were added to the reaction mixture (exposure time ~ 5 min at 24°C).

Fig. 6. Inhibition of dephosphorylation of EGFR by purified RPTP α . Autophosphorylated EGFR was incubated with M6 buffer (pH 6.0) in the absence (control) or presence of $1 \mu\text{g/ml}$ purified RPTP α , without or with $20 \mu\text{M}$ of PLC γ -SH2 (construct V) for 30 min at 37°C . Samples were then analyzed on 8% SDS-PAGE gels (exposure time ~ 5 min at 25°C).

ditions (pH 6.0) and at a concentration of $1 \mu\text{g/ml}$, partially dephosphorylated the EGFR. This dephosphorylation was prevented by $20 \mu\text{M}$ PLC γ -SH2 (construct V) (Figure 6), with an IC_{50} of inhibition of dephosphorylation of ~ 2 – $5 \mu\text{M}$ (not shown). It is noteworthy that unlike the dephosphorylation of the EGFR by A431 or HER14 phosphatases, dephosphorylation by RPTP α was incomplete. Indeed, preliminary mapping experiments revealed that one of the EGFR autophosphorylation sites was not dephosphorylated by RPTP α (Rotin *et al.*, unpublished

Table I. Dephosphorylation of RCML^a and MBP^a by RPTP α ^b in the absence (buffer) or presence of GST^c or PLC γ -SH2^c

Substrate	³² P _i released (%)		
	Buffer	GST	PLC γ -SH2
RCML (10–15 μ M)	100	117.3 ($\pm$ 5.2)	153.3 ($\pm$ 16.4)
MBP (2–6 μ M)	100	113.0	112.5

As variation in the concentrations of RCML, MBP, GST, PLC γ -SH2 and RPTP α within the indicated ranges did not affect the results, data from all experiments were pooled. The data represent mean values of duplicate determinations from 2–4 separate experiments. Numbers in parentheses represent standard errors calculated where applicable.

^aRCML, reduced carboxamidomethylated and maleylated lysozyme; MBP, myelin basic protein.

^bRPTP α concentrations were 3–13 μ g/ml for RCML and 5–330 μ g/ml for MBP. Enzymatic activity of RPTP α was assayed in M6 buffer (pH 6.0).

^cThe concentrations of GST and PLC γ -SH2 (constructs I or V) were 5–10 μ M.

results). This suggests that tyrosine phosphatases may preferentially dephosphorylate specific residues of phosphotyrosine-containing proteins.

To determine the specificity of the SH2-mediated inhibition of dephosphorylation by RPTP α , we analyzed the effect of PLC γ -SH2 on dephosphorylation by RPTP α of tyrosine-phosphorylated artificial substrates. Tyrosine-phosphorylated reduced carboxamidomethylated and maleylated lysozyme (RCML) and myelin basic protein (MBP) were used as substrates in these assays, which measure the amount of ³²P_i released by the activity of the phosphatase (Tonks *et al.*, 1988a,b). The results (Table I) show that PLC γ -SH2 (constructs I or V) did not inhibit the dephosphorylation (i.e. ³²P_i release) of RCML or MBP by RPTP α . This suggests that the SH2-mediated inhibition of dephosphorylation is specific and probably occurs only at tyrosine residues located in SH2 binding regions.

Previous work from our laboratory (Margolis *et al.*, 1990b) has localized the binding region of PLC γ -SH2 to the autophosphorylated carboxy-terminal tail of the EGFR. However, the specific tyrosine phosphorylation site(s) to which PLC γ -SH2 binds has not been identified. Since we found complete correlation between the ability of the SH2 domains to bind to the receptor and to inhibit its dephosphorylation, we have utilized the SH2-mediated protection of dephosphorylation as a simple approach to map the exact binding site(s) of the EGFR to PLC γ -SH2. Hence, the EGFR was immunoprecipitated from A431 or HER14 cells, phosphorylated with [γ -³²P]ATP and dephosphorylated in the presence of increasing concentrations of construct V (2SH2) or construct VII (C-SH2). Following separation on SDS-PAGE and transfer to nitrocellulose, the band corresponding to the EGFR was excised and trypsin digested. The resulting fragments were then separated on HPLC and the radioactivity of the collected fractions counted. As shown in Figure 7A, trypsin-digested autophosphorylated EGFR was separated into five peaks, representing the previously mapped tyrosine phosphorylation sites Y1173, Y1086, Y1148 (Downward *et al.*, 1984), Y1086 (Margolis *et al.*, 1989b) and Y992 (Walton *et al.*, 1990). Our own identification of Y992 was carried out by trypsin digesting phosphorylated EGFR and purifying the phosphotyrosine-containing fragments with anti-phosphotyrosine antibodies immobilized on beads, followed by

Fig. 7. Protection from dephosphorylation of specific tyrosine phosphorylation sites of the EGFR by PLC γ -SH2. Phosphorylated EGFR were dephosphorylated with A431 cell lysate in the presence of the indicated concentrations of PLC γ -2SH2 (construct V) or PLC γ -C-SH2 (construct VII). Following separation on 7% SDS-PAGE gels, transfer to nitrocellulose and trypsin digestion, the ³²P-labeled tryptic peptides were separated by HPLC. (A) ³²P-labeled tryptic peptides from control (not dephosphorylated) EGFR, showing separation of the five peaks corresponding to Y1173, Y1086, Y1148, Y1068 and Y992. (B) Tryptic peptides from dephosphorylated (lysate-treated) EGFR. (C–F) As in B, only the dephosphorylation was carried out in the presence of 1 μ M (panel C), 6 μ M (panel D), 30 μ M (panel E) or 100 μ M (panel F) PLC γ -2SH2 (construct V). (G) As in B, only dephosphorylation was carried out in the presence of 30 μ M PLC γ -C-SH2 (construct VII). A maximum of 50 000 c.p.m. were used for each HPLC analysis and the radioactivity of each fraction was multiplied by the appropriate factor to obtain total c.p.m.

Fig. 8. Identification of the tyrosine autophosphorylation site Y992. EGFR was immunoprecipitated from A431 cells and subjected to autophosphorylation as described in Materials and methods. Following separation on 7% SDS-PAGE gels, transfer to nitrocellulose and trypsin digestion, the ^{32}P -labeled peptides were purified with anti-phosphotyrosine antibodies immobilized on beads, followed by HPLC. The tryptic peptide eluted at 39.5% acetonitrile was then microsequenced. The resulting partial sequence (ALMDEEDMDDVVDA) identifies it as the peptide containing Y992. Partial sequencing of the peaks eluted at 36% acetonitrile and 37.5% acetonitrile reconfirms the previous assignment of Y1148 and Y1068, respectively.

HPLC. The peptide eluting at 39.5% acetonitrile was then directly sequenced and identified as the peptide containing the phosphorylated Tyr992 (Figure 8). Dephosphorylation of autophosphorylated EGFR with A431 cell lysate led to the dephosphorylation of all tyrosine-phosphorylated sites (Figure 7B). However, in the presence of increasing concentrations of PLC γ -2SH2 (construct V), the dephosphorylation of each phosphorylation site was differentially inhibited (Figure 7C–F). At 1 μM PLC γ -2SH2, the primary site protected from dephosphorylation was Y992, but a weak protection of Y1068 was observed as well. Y1173 was also protected at 6 μM and at 100 μM partial protection of Y1086 was observed. No protection of Y1148 was observed at PLC γ -2SH2 concentrations as high as 100 μM . Thus, the hierarchy of protection from dephosphorylation was Y992 > Y1068 > Y1173 > Y1086. These results demonstrate that Y992, as confirmed by direct sequencing, is the high-affinity binding site of the EGFR towards PLC γ -SH2. This conclusion is further supported by the fact that the tryptic peptide which contains the phosphorylated Y992 was able to bind to immobilized PLC γ -SH2 (construct V) domains, but not to immobilized control PLC γ -SH3 (construct III) domain (Figure 9). Interestingly, PLC γ -C-SH2 (construct VII) required a much higher concentration (30 μM) to achieve the same level of protection from dephosphorylation as seen with $\sim 6 \mu\text{M}$ PLC γ -2SH2 (construct V) (compare Figure 7E to G).

Discussion

The results presented in this report provide evidence that recombinant SH2 domains derived from PLC γ or GAP prevent tyrosine dephosphorylation of receptors with tyrosine kinase activity. This is a novel function of the SH2 domain,

Fig. 9. Binding of the phosphorylated tryptic peptide containing Y992 to PLC γ -SH2. Phosphorylated EGFR was dephosphorylated in the presence of 3 μM PLC γ -2SH2 (construct V). It was then separated on 7% SDS-PAGE gels, transferred to nitrocellulose and the EGFR band digested with trypsin and purified by HPLC. The tryptic peptide containing the phosphorylated Y992 (10 500 c.p.m.) was dried, resuspended in HNTG, divided in half and incubated with glutathione-agarose beads bound to either 1 μM PLC γ -SH2 (construct V) or 1 μM PLC γ -SH3 (construct III). Following washes, the samples were analyzed by SDS-PAGE (15–20%) and autoradiography (exposure time 5 h at -80°C).

which may help explain the mechanisms underlying the transforming capacity of the oncogene *v-crk*. The product of *v-crk* was previously shown to form a complex with several tyrosine-phosphorylated proteins and to elevate the phosphotyrosine content of transformed cells (Matsuda *et al.*, 1990, 1991; Mayer and Hanafusa, 1990). It is possible therefore that *v-crk* causes transformation by binding to a certain class of tyrosine-phosphorylated target proteins, thereby preventing their dephosphorylation by tyrosine-specific phosphatases. Several other SH2-containing proteins, such as GAP (Moran *et al.*, 1990) or the *abl* gene product (Mayer *et al.*, 1991), were also shown to form complexes with tyrosine-phosphorylated target proteins.

Our results showing a correlation between the ability of the SH2-containing constructs to bind to the EGFR and their ability to prevent its dephosphorylation (Figure 2) suggest that the SH2 domain and tyrosine phosphatases compete for the autophosphorylated carboxy-terminal tail of the receptor. These findings are also consistent with the fact that the SH2 domain of P85 binds to a tyrosine-phosphorylated region within the kinase insert of PDGFR (Kazlauskas and Cooper, 1989; Escobedo *et al.*, 1991b). It is not clear yet whether SH2 domains are able to interact with and prevent tyrosine dephosphorylation of cellular substrates. Moreover, a direct interaction with tyrosine phosphatases cannot be ruled out. However, the data in Figure 6 show that EGFR dephosphorylation by a purified tyrosine phosphatase (RPTP α) is prevented by PLC γ -SH2. Interestingly, tyrosine dephosphorylation of artificial tyrosine-phosphorylated substrates, such as RCML or MBP (Table I), was not prevented by the SH2-containing recombinant proteins. These results provide further support for the notion that the inhibition of receptor dephosphor-

ylation by SH2 domains involves direct competition with the tyrosine phosphatase moiety. Since tyrosine autophosphorylation of growth factor receptors is involved in the regulation of receptor activity, this inhibition may provide a regulatory function.

The degree of SH2-mediated inhibition of dephosphorylation of the EGFR was dose dependent (Figure 3). Moreover, our results (Figure 7) demonstrate the specificity and hierarchy of this inhibition towards the different tyrosine phosphorylation sites of the receptor: Y992 > Y1068 > Y1173 $\gg$ Y1096. No inhibition of Y1148 was observed in our experiments. These findings therefore implicate Y992 as the high-affinity binding site of the EGFR to PLC γ -SH2. Moreover, they also demonstrate that the ability of SH2-containing protein to inhibit tyrosine dephosphorylation of specific residues can be used as a simple novel technique to map the binding sites of the tyrosine-containing proteins to SH2-containing proteins. Close analysis of the region surrounding Y992 and Y1068, which binds PLC γ -SH2 with high affinity, reveals a common motif: V/LXXXXEYL/I. The EYL/I sequence is also found around Y1173. The common motif is not found around Y1148 or Y1086, which either does not bind or binds poorly to PLC γ -SH2, respectively. Interestingly, we have recently reported that a similar sequence motif in the FGF receptor (*flg*) (LXXXXEYL) also functions as a major binding site for PLC γ -SH2 (Mohammadi *et al.*, 1991). Thus, it appears that the tyrosine-phosphorylated sequence motif V/LXXXXEYL/I may constitute a high-affinity binding site for the SH2 domains of PLC γ .

The ability of two SH2 domains (construct V) to inhibit EGFR dephosphorylation better than one SH2 domain (construct VII) (see Figure 7E versus G) is consistent with previous reports which show greater binding capacity to the EGFR of recombinant PLC γ -SH2-SH2 than PLC γ -SH2 (Anderson *et al.*, 1990), and with our own recent work showing that the presence of two SH2 domains in recombinant fragments of PLC γ lowers the K_m of EGFR-mediated substrate phosphorylation relative to one SH2 domain (Rotin *et al.*, 1992).

The results presented in Figure 5A show a clear inhibition of PDGFR dephosphorylation by PLC γ -SH2 (construct I). It is noteworthy that previous studies have shown that a major tyrosine autophosphorylation site of PDGFR (Tyr751) which is located in the kinase insert region is not a binding site for PLC γ (Kazlauskas and Cooper, 1989; Escobedo *et al.*, 1991b). Thus, only partial protection of PDGFR dephosphorylation by PLC γ -SH2 was expected. It is possible that lower doses of PLC γ -SH2 than the one used in our experiments would have yielded only partial inhibition of dephosphorylation of the PDGFR. Further analysis is required in order to determine the specificity of the various SH2-containing signaling molecules towards the different autophosphorylation sites of the PDGFR, and their ability to protect the dephosphorylation of the various tyrosine-phosphorylated sites.

It is possible that the inhibition of tyrosine dephosphorylation by the SH2 domains of GAP or PLC γ is important to the physiological function of these molecules, especially since we observed such inhibition at PLC γ -2SH2 concentrations as low as 1 μ M, which are within the physiological range of PLC γ concentrations. If binding of these molecules to growth factor receptors is important in their activation, then prevention of dephosphorylation of the receptor would be

crucial to maintain binding. However, we have demonstrated that the tyrosine phosphorylation of PLC γ reduces its affinity for EGFR and this may reduce its ability to inhibit dephosphorylation in the living cell (Margolis *et al.*, 1990a). The true physiological function of the SH2 domain binding may be to target substrate molecules to autophosphorylated tyrosine kinases and thereby determine selectivity of the signal transduction pathways. In separate experiments, we have compared the kinetic parameters of tyrosine phosphorylation by the EGFR of the various constructs depicted in Figure 1. In brief, it appears that the presence of SH2 domains decreases by $\sim$ 40-fold the apparent K_m of substrate tyrosine phosphorylation (Rotin *et al.*, 1992). These results suggest that the tyrosine-phosphorylated carboxy-terminal tail of the EGFR, which is the binding region for the SH2 domain of PLC γ , GAP (Margolis *et al.*, 1990b) and other SH2-containing proteins, such as p85 (Skolnik *et al.*, 1991) promotes the targeting of substrates by increasing their affinity towards the cytoplasmic catalytic domain. However, when certain SH2 domains are over-expressed, such as in *v-crk*-transformed cells, this binding to tyrosine-phosphorylated residues results in an increase in cellular tyrosine phosphorylation by blocking phosphatase action. Hence, the normal physiological role of SH2 domains is probably to target signaling molecules for binding to activated growth factor receptors and other tyrosine-phosphorylated proteins [reviewed in Koch *et al.* (1991)]. However, upon aberrant expression or overexpression the SH2 domains may have a pathological role by increasing cellular tyrosine phosphorylation, leading to oncogenesis. Clearly, the results shown in this report provide further support for the crucial regulatory role of SH2 domains in signal transduction.

Materials and methods

Reagents and solutions

The pGEX-3X plasmid was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden), isopropyl- β -D-thiogalactopyranoside (IPTG) was from USB Corp. (Cleveland, OH). [γ - 32 P]ATP was from DuPont (Boston, MA), restriction endonucleases and trypsin were from Boehringer Mannheim (Indianapolis, IN), anti-phosphotyrosine antibody beads were from Oncogene Science (Manhasset, NY). Tissue culture media and reagents were from Gibco (New York), and EGF and PDGF were from Intergen (New York). All other chemicals were from Sigma (St Louis, MO). All reagents were of analytical grade. HNTG solution contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol. Lysis buffer contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl $_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μ g/ml aprotinin and 10 μ g/ml leupeptin. HEPES solution contained 20 mM HEPES (pH 7.5). M6 buffer contained 20 mM MES (pH 6.0), 5% glycerol, 0.1% β -mercaptoethanol and 0.1% Triton X-100.

Cells

A431 cells (expressing $\sim 2 \times 10^6$ EGFR/cell) and HER14 cells (NIH3T3 cells expressing $\sim 4 \times 10^5$ human EGFR/cell) were maintained in Dulbecco's modified Eagle medium supplemented with 50 μ g/ml streptomycin, 50 U/ml penicillin, 2 mM glutamine and 10% fetal calf serum (A431 cells) or calf serum (HER14 cells). To obtain cell lysate, confluent 15 cm plates ($\sim 2.5 \times 10^7$ cells) were lysed in 1 ml lysis buffer, the debris pelleted and the supernatant (containing EGFR and phosphatases) frozen until use.

Production of recombinant fusion proteins

Oligonucleotides homologous to the boundaries of the desired sequence within the human PLC γ cDNA (Burgess *et al.*, 1990) were synthesized. These oligonucleotides contained the *Bam*HI and *Eco*RI restriction sites. The required stretch of DNA was subsequently amplified by PCR using human PLC γ cDNA as a template. The DNA insert was then digested with *Bam*HI and *Eco*RI, and ligated into the pGEX-3X bacterial expression plasmid.

The recombinant plasmid was introduced into *E. coli* and the bacterial transformants analyzed for the presence of insert. The GST insert fusion protein was then expressed by induction with 1 mM IPTG. Expressed GST fusion proteins were isolated from bacterial lysates by affinity chromatography with glutathione-agarose beads, followed by elution with 7.5 mM reduced glutathione.

Immunoprecipitations

The EGFR was immunoprecipitated from A431 cell lysate ($\sim 5-10 \times 10^{12}$ receptors/assay) using monoclonal antibodies (mAb108) bound to protein A-Sepharose, as described previously (Honegger *et al.*, 1988). To immunoprecipitate the PDGFR, HER14 (NIH3T3) cells were serum starved for 20 h in 0.5% calf serum. The cells were lysed and samples incubated with 10 ng/ml PDGFR for 30 min on ice. The stimulated lysate was then added onto protein A-Sepharose beads previously incubated with anti-PDGFR antibodies and immunoprecipitation proceeded for 90 min at 4°C.

Phosphorylation and dephosphorylation assays

Following immunoprecipitation, immobilized EGFR were autophosphorylated in the presence of 10 μ Ci [γ - 32 P]ATP (per assay), 4.5 μ M unlabeled ATP and 13 mM MnCl_2 , as detailed previously (Honegger *et al.*, 1988). The tyrosine-phosphorylated EGFR complex bound to the beads was washed (four times) with ice-cold 20 mM HEPES solution (pH 7.5) and aliquoted evenly into separate tubes (0.1 μ M EGFR/assay). Dephosphorylation was subsequently carried out either with 100 μ l 20 mM HEPES solution (control) or with 80 μ l A431 lysate (cells previously lysed with lysis buffer) plus 20 μ l HEPES solution, either alone or with the indicated concentrations of the appropriate construct (I–VII), the GST fusion sodium protein alone, or with 200 μ M sodium orthovanadate, for 30 min at 37°C. The reaction was stopped by adding Laemmli sample buffer. Proteins were then separated by SDS-PAGE and autoradiographed to demonstrate the presence/absence of phosphorylated EGFR. Exactly the same protocol was carried out for the PDGFR, except that autophosphorylation was performed with 10 μ Ci [γ - 32 P]ATP, 2 μ M unlabeled ATP and 10 mM MnCl_2 , and HER14 cell lysate was utilized as a source of phosphatases.

For measuring dephosphorylation of the EGFR by the receptor type RPTP α (Kaplan *et al.*, 1990b), which was expressed in insect cells and purified by Daum *et al.* (1991), the EGFR was immunoprecipitated and autophosphorylated as described above. It was then dephosphorylated in 100 μ l M6 buffer (pH 6.0) in the absence (control) or presence of 100 ng RPTP α /assay (1 μ g/ml), either without or with 20 μ M PLC γ -SH2 (construct V). RPTP α activity was assayed at pH 6.0 because its enzymatic activity towards tyrosine-phosphorylated substrates was optimal at this pH (Daum *et al.*, 1991).

To determine phosphatase activity towards artificial substrates, RCML and MBP were tyrosine-phosphorylated with [γ - 32 P]ATP as described previously (Tonks *et al.*, 1988a,b). Then 10–15 μ M RCML or 2–6 μ M MBP were incubated in 60 μ l of M6 buffer (pH 6.0), in the presence of RPTP α (at 3–13 μ g/ml for RCML and 5–330 μ g/ml for MBP) and in the absence (buffer alone) or presence of 5–10 μ M GST alone or 5–10 μ M GST-PLC γ -SH2 (construct I or V) for 10 min at 30°C. The reaction was stopped by adding 180 μ l of 20% trichloroacetic acid and, following centrifugation, 200 μ l of the supernatant were counted in a β -counter to determine the amount of $^{32}\text{P}_i$ released by the phosphatase. Because differences in the concentrations of RCML, MBP, RPTP α , GST and GST-PLC γ -SH2 within the indicated ranges did not affect the outcome of the experiment, the data from all determinations, each performed in duplicate, were pooled.

Trypsin digestion, peptide purification and microsequencing

Immunoprecipitated EGFRs from either A431 or HER14 cells were phosphorylated as described above. Following dephosphorylation in the presence of various concentrations of constructs V or VII, the EGFRs were purified on 7% SDS-PAGE gels, transferred to nitrocellulose and the radioactive bands corresponding to the EGFR excised and their radioactivity determined by Cerenkov counting. The nitrocellulose slices were then incubated for 30 min (37°C) with 0.5% polyvinylpyrrolidone in 100 mM acetic acid, washed thoroughly and incubated with 20 μ g/ml trypsin in 50 mM ammonium bicarbonate (pH 8.3–8.5) overnight at 37°C. The resulting peptides were purified on HPLC using a C $_{18}$ reversed-phase column with 0.1% trifluoroacetic acid in H_2O (buffer A) or in acetonitrile (buffer B). The following gradient was applied at a flow rate of 1 ml/min: 0–10 min, 0% CH_3CN ; 10–15 min, linear gradient to 40% CH_3CN ; 50–50.1 min, increase to 60% CH_3CN . For peptide sequencing, $\sim 1 \times 10^{15}$ EGFR (~ 300 μ mol) from A431 cells were phosphorylated and trypsin digested as described above. The resulting fragments were then incubated with anti-phosphotyrosine antibody beads and soya bean trypsin

inhibitor (10 μ g/ml) in 50 mM NaCl plus 50 mM HEPES solution (pH 7.5). Following thorough washes, the tyrosine-phosphorylated fragments were eluted with 50 mM phenyl phosphate and separated by HPLC as described above. The appropriate ^{32}P -labeled peptides were then analyzed by a protein microsequencer (Applied Biosystems, model 447A protein sequencer with an on-line model PTH analyzer). For binding of phosphorylated tryptic peptides, the HPLC-purified fragments were dried in a speed vacuum and redissolved in HNTG.

Receptor binding assays

To test for association of the GST-SH2/SH3 constructs with the EGFR, HER14 cells were starved in 1% calf serum overnight and incubated with either phosphate-buffered saline (PBS) (control) or with 40 nM EGF for 2 min at 37°C. Cells were then lysed in lysis buffer containing 100 mM NaF, 30 mM *p*-nitrophenyl phosphate, 10 mM pyrophosphate and 200 μ M sodium orthovanadate. Approximately 5–10 μ g of the indicated constructs, bound to glutathione-agarose beads, were incubated for 2 h at 4°C with 800 μ l of the above lysates. They were then washed in HNTG solution and resuspended in Laemmli sample buffer. Proteins were separated on 8% SDS-PAGE gels, followed by immunoblotting with antibodies directed against the carboxy terminus (anti-C) of the EGFR.

Acknowledgements

We thank C.A.Koch and T.Pawson for GAP cDNA. D.R. is supported by a fellowship from the Medical Research Council of Canada. B.M. is a Lucille P.Markey scholar. R.D. is the recipient of a travel fellowship from the Imperial Cancer Research Fund. G.D. is supported by a fellowship from the Deutsche Forschungsgemeinschaft. This work is supported by a grant from Rorer Central Research (J.S.), by a grant from the Human Frontier Science Program (HFSP) (J.S.), by a grant from the Lucille P.Markey Trust (B.M.), by NIH grants DK0709 and GM42508 (E.H.F.), and by a grant from the Muscular Dystrophy Association of America (E.H.F.).

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Received on May 13, 1991; revised on November 11, 1991