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13. Procedures for preparation of *X. laevis* oocytes, voltage-clamp recording, and flash photolysis were as described [*10*; K. Sumikawa, I. Parker, R. Miledi, *Methods Neurosci.* **1**, 30 (1989)]. Oocytes were loaded intracellularly with ~1 pmol of caged $InsP_3$ (*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl) ethyl ester; CalBiochem) and ~0.5 pmol of Fluo-3 or Rhod-2 (Molecular Probes). Optical measurements were made from restricted regions of the vegetal hemisphere to avoid light absorption by pigment in the animal hemisphere. Two systems were used to monitor intracellular $Ca^{2+}$ with long wavelength fluorescent indicators (*12*) to minimize photolysis of caged $InsP_3$. Both were constructed from an upright Zeiss microscope fitted with two stacked epifluorescence units. The lower epifluorescence unit provided flashes of near UV light for photolysis, and the upper provided fluorescence excitation for the $Ca^{2+}$ indicator. In the first system (*4*), Fluo-3 fluorescence was recorded from a relatively large area (~$10^4$ $\mu m^2$) and coincident with the photolysis light. The second system used confocal optics to monitor $Ca^{2+}$-dependent fluorescence from a virtual point source in the cytoplasm. Light from a 0.2 mW green (543.5 nm) He-Ne laser was focused by a secondary lens in the epifluorescence unit and reimaged as a diffraction-limited spot by a 40× water immersion objective (numerical aperture, 0.75), about 5 μm below the surface of the oocyte. Emitted light at wavelengths > 590 nm was collected through the same lens and monitored by a photomultiplier through a 50 μm pinhole positioned confocally in the microscope photo-tube. The photolysis light was focused as a square (area, 50 to 100 $\mu m^2$) centered around the monitoring spot. Rhod-2 was used as the indicator in these experiments, as its excitation spectrum matches well the emission of the inexpensive He-Ne laser. From the size of the detector pinhole and the magnification of the objective lens, we estimated that signals were recorded from a spot with a diameter of about 2 μm, in the plane of the membrane. Measurements obtained by focusing the microscope through a thin (~1 μm) film of rhodamine solution further indicated that the signal was largely restricted to a depth of about 10 μm in the cytoplasm. The monitoring spot remained fixed and was not scanned, as in confocal imaging microscopy. Increases in fluorescence of both Fluo-3 and Rhod-2 corresponded to increasing free $Ca^{2+}$, but because neither shows spectral shifts with $Ca^{2+}$-binding, we did not calibrate signals in terms of free $Ca^{2+}$ concentration.
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# Binding of SH2 Domains of Phospholipase $C_\gamma 1$, GAP, and Src to Activated Growth Factor Receptors

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**Phospholipase $C_\gamma 1$ ($PLC_\gamma 1$) and $p21^{ras}$ guanosine triphosphatase (GTPase) activating protein (GAP) bind to and are phosphorylated by activated growth factor receptors. Both $PLC_\gamma 1$ and GAP contain two adjacent copies of the noncatalytic Src homology 2 (SH2) domain. The SH2 domains of $PLC_\gamma 1$ synthesized individually in bacteria formed high affinity complexes with the epidermal growth factor (EGF)- or platelet derived growth factor (PDGF)-receptors in cell lysates, and bound synergistically to activated receptors when expressed together as one bacterial protein. In vitro complex formation was dependent on prior growth factor stimulation and was competed by intracellular $PLC_\gamma 1$. Similar results were obtained for binding of GAP SH2 domains to the PDGF-receptor. The isolated SH2 domains of other signaling proteins, such as $p60^{src}$ and Crk, also bound activated PDGF-receptors in vitro. SH2 domains, therefore, provide a common mechanism by which enzymatically diverse regulatory proteins can physically associate with the same activated receptors and thereby couple growth factor stimulation to intracellular signal transduction pathways.**

A VARIETY OF POLYPEPTIDE HORmones that elicit cell growth and differentiation bind to cell-surface receptors with intracellular protein-tyrosine kinase domains (*1*). Growth factors apparently activate their receptors by inducing receptor dimerization and subsequent autophosphorylation on tyrosine, evoking a catalytically active receptor capable of phosphorylating cellular substrates (*1–3*). Activated EGF- and PDGF-receptors (EGF-R; PDGF-R) complex with a set of cytoplasmic proteins that directly regulate intracellular signal transduction pathways. These include the $_\gamma 1$ isoform of the phosphoinositide-specific phospholipase C (PLC) (*4, 5*), $p21^{ras}$ GTPase activating protein (GAP) (*6, 7*), phosphatidyl inositol (PI) 3′-kinase (*8*), and $p74^{raf}$ (*9*). These results suggest that critical targets for receptor tyrosine phosphorylation are selected from the pool of potential substrates by their ability to physically complex with the receptor. A simple mechanism to accomplish these interactions would be the provision of cytoplasmic ligands with a common structural domain that recognizes autophosphorylated receptors.

The proteins that associate with activated growth factor receptors have quite distinct enzymatic properties and are structurally unrelated within their catalytic domains. However, $PLC_\gamma 1$ (*10*) and GAP (*11*) each contain two adjacent copies of a noncatalytic domain of ~100 amino acids, called the Src homology (SH) region 2 (*12*) (Fig. 1). The SH2 domain was first identified in nonreceptor protein tyrosine kinases like Src and Fps, by its apparent ability to interact with the kinase domain and phosphorylated substrates (*13–15*). An SH2 sequence has also been identified in the v-Crk oncoprotein, which complexes with several tyrosine phosphorylated proteins in *crk*-transformed cells (*16*). Most SH2-containing proteins also contain a motif, SH3, which is found independently in several cytoskeletal proteins and may mediate interactions with the cytoskeleton (*12, 16, 17*). The SH2 domains have been implicated in protein-protein interactions that involve protein-tyrosine kinases and their substrates (*13, 15*). This raises the possibility that enzymes such as $PLC_\gamma$ and GAP associate directly with activated tyrosine kinase receptors by virtue of their SH2 domains (*18*).

To test this hypothesis, restriction sites were introduced into the complementary DNA (cDNA) for bovine $PLC_\gamma 1$, which allowed the precise excision of the $NH_2$-terminal and COOH-terminal SH2 domains (SH2[N] and SH2[C]), either alone or together (Fig. 1) (*19*). The individual SH2 domains, or the two SH2 domains together (SH2[N + C]) were introduced into a bacterial expression vector (pATH) and expressed as TrpE fusion proteins in *Escherichia coli*. These proteins were isolated from bacterial lysates by immunoprecipitation with antibodies to TrpE (anti-TrpE) attached to Sepharose beads (*20*). The immobilized bacterial proteins were then incubated with lysates of either Rat-1 cells that expressed the human EGF-R, which had been stimulated with EGF or Rat-2 cells that expressed the PDGF-R, which had been stimulated with PDGF. The immunoprecipitates were recovered, washed extensively, and analyzed for associated phosphotyrosine (P.Tyr)–containing proteins by immunoblotting with antibodies to P.Tyr (anti-P.Tyr) (Fig. 2). The TrpE-PLC-

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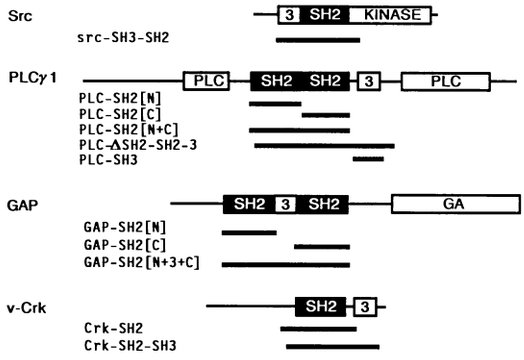


**Fig. 1.** Locations of SH2 and SH3 domains. The solid bars beneath the proteins indicate the regions expressed as bacterial TrpE fusion proteins for in vitro binding experiments (*32*). Abbreviations: 3, SH3 domain; PLC, catalytic regions of $PLC_\gamma1$; GA, GTPase activating region of GAP; kinase, Src tyrosine kinase domain.

SH2[N] fusion protein complexed specifically with a 180-kilodalton (kD) P.Tyr-containing protein in lysates of EGF-stimulated cells. Immunoblotting of duplicate samples with antibodies to the EGF-R confirmed that this protein was the EGF-R and showed that its in vitro association with the $PLC_\gamma1$ SH2[N] domain was EGF-dependent (Fig. 2). The $PLC_\gamma1$ SH2[N] domain was more efficient than the SH2[C] domain in its ability to bind the EGF-R. Interestingly, the fusion protein that contained both $NH_2$- and COOH-terminal SH2 domains bound two to four-fold more EGF-R in EGF-stimulated cell lysates than could be accounted for by the two individual SH2 domains. The $PLC_\gamma1$ SH2 domains therefore functioned synergistically in binding to the activated EGF-R. Very similar results were obtained for interactions of the $PLC_\gamma1$ SH2 domains with the PDGF-R (Fig. 2). The $PLC_\gamma1$ SH2[N] domain bound the PDGF-R in lysates of cells treated with the BB homodimeric form of PDGF but not in lysates of unstimulated cells. As observed for the EGF-R, the $PLC_\gamma1$ SH2[C] domain alone was inefficient in binding activated PDGF-R, but bound synergistically with the SH2[N] domain when both domains were expressed as one bacterial protein (Fig. 2).

Within the SH2 domain, there are motifs that are particularly highly conserved. For example the $NH_2$-terminal tryptophan is invariant, and most SH2 domains start with the consensus W(Y,F)(H,F)GK (*15, 21*). A likely possibility is that these residues have been conserved because they are important in the interactions of SH2-containing proteins with activated growth factor receptors. We therefore expressed a TrpE fusion protein that contained both $PLC_\gamma1$ SH2 domains, with the exception that the first four residues of SH2[N] (W-F-H-G) were deleted (PLC ΔSH2-SH2-3). This fusion protein showed a modest ability to bind activated EGF- or PDGF-R (Fig. 2, lanes 5 and 10) that was equivalent to the SH2[C] domain alone, indicating that the removal of the four residues weakened binding activity.

Because GAP also associates with the PDGF-R, we undertook similar experiments using bacterial GAP SH2 sequences (see Fig. 1). The GAP SH2[N] domain bound the PDGF-R in a lysate of PDGF-stimulated cells (Fig. 3), but not in unstimulated cells (*22*). The GAP SH2[C] domain exhibited much weaker PDGF-R–binding activity. However, the two SH2 domains together (GAP-SH2[N + 3 + C] bound the receptor threefold more efficiently than expected from their individual binding activities (Fig. 3, lanes 4 to 6 and 13 to 15). GAP contains an SH3 domain, which intervenes between the two SH2 elements and might contribute to binding to receptors. This seems unlikely, because the $PLC_\gamma1$ SH3 domain, expressed in isolation as a TrpE fusion protein, did not associate with the PDGF-R (Fig. 3).

Only a minor fraction of activated PDGF-R complexes with $PLC_\gamma1$ in vivo. We genetically modified a Rat-2 cell line to overexpress $PLC_\gamma1$ by tenfold as compared with the endogenous enzyme (Rat-2 $PLC_\gamma1$) (*23*). There is a proportionate increase in the amount of PDGF-R precipitated with antibodies to $PLC_\gamma1$ (anti-$PLC_\gamma1$) after PDGF stimulation of Rat-2 $PLC_\gamma1$ cells, in comparison with parental Rat-2 cells (*23*). If bacterial $PLC_\gamma1$ SH2 domains bound to the same site(s) on the PDGF-R as did cellular $PLC_\gamma1$, then overexpression of $PLC_\gamma1$ should block binding of bacterial $PLC_\gamma1$ SH2 domains to activated PDGF-R in vitro. Consistent with this prediction, when the Rat-2 $PLC_\gamma1$ cell line was stimulated with PDGF, lysed, and incubated with immobilized $PLC_\gamma1$-SH2[N] or $PLC_\gamma1$ SH2[N + C], only one-third as much PDGF-R associated with the bacterial protein, compared with the parental PDGF-stimulated Rat-2 cells (Fig. 4). Binding of TrpE-GAP-SH2 fusion protein to the PDGF-R was also reduced by overexpression of endogenous $PLC_\gamma1$, suggesting that $PLC_\gamma1$ and GAP compete for sites on the activated PDGF-R.

Src-like tyrosine kinases and v-Crk also contain SH2 domains, which may bind activated receptors. Consistent with this prediction, bacterial fusion proteins that contained the SH2 domains of $p60^{src}$ or $P47^{gag\text{-}crk}$ bound PDGF-R in lysates of PDGF-stimulated Rat-2 cells (Fig. 3). $p60^{src}$ is a substrate for the PDGF-R (*24*), and recent evidence suggests that Src-like kinases are physically associated with activated PDGF-R in vivo (*25*). Our data imply that this interaction involves the Src SH2 domain. Whether the normal homolog of v-Crk complexes with growth factor receptors in vivo remains to be established.

These results indicate that individual $PLC_\gamma1$ or GAP SH2 domains, isolated as bacterial fusion proteins, can form stable complexes with activated growth factor receptors. The 100 amino acids of the $PLC_\gamma1$ and GAP SH2 domains therefore contain sufficient structural information to form independent, high affinity binding sites for receptors. The ability of SH2 domains to form such complexes when fused to a heterologous TrpE polypeptide suggests that SH2 domains may act in vivo to physically couple enzymatically diverse signaling proteins such as $PLC_\gamma$ and GAP to growth

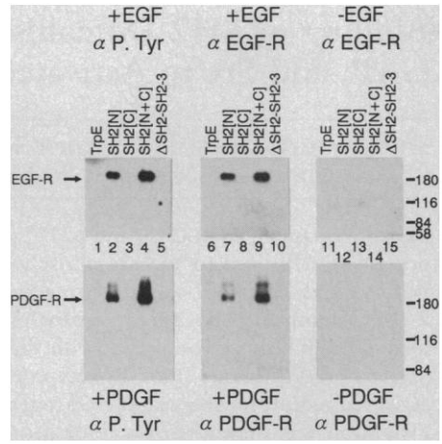


**Fig. 2.** SH2 domains of $PLC_\gamma1$ synthesized in bacteria bind synergistically in vitro to activated EGF- and PDGF-receptors. (**A**) Immobilized parental TrpE or the indicated TrpE-$PLC_\gamma1$ bacterial fusion proteins were incubated with lysates of Rat-1 cells that overexpressed the human EGF-R (R1hER), which had been serum-starved for 48 hours (lanes 11 to 15) or stimulated for 5 min at 37°C with 80 nM EGF (lanes 1 to 10). Complexes were washed, resolved on 8.25% SDS-polyacrylamide gels, and analyzed by immunoblotting with either anti(α)-P.Tyr (lanes 1 to 5) or anti-EGF-R (lanes 6 to 15) followed by $I^{125}$-labeled protein A. Autoradiography was for 18 hours. (**B**) Immobilized TrpE or TrpE-$PLC_\gamma1$ fusion proteins, as in (A), were incubated with lysates from Rat-2 cells that were serum-starved for 48 hours (lanes 11 to 15) or stimulated for 5 min at 37°C with 75 nM BB-PDGF (lanes 1 to 10). Samples were resolved on 6% SDS-polyacrylamide gels and analyzed by immunoblotting with either anti-P.Tyr (lanes 1 to 5) or anti-PDGF-R (lanes 6 to 15).

factor receptors. Sequences that are structurally and functionally similar to the catalytic domains of PLC$_\gamma$1 and GAP have been identified in proteins that do not have SH2 domains. Several distinct isoforms of PLC have been isolated, including PLC$_\beta$ and PLC$_\delta$, which share enzymatic sequences with PLC$_\gamma$, but lack the central PLC$_\gamma$ SH2 and SH3 domains (*26*). Only the PLC$_\gamma$1 isoform has been shown to complex with growth factor receptors (*4*) or to stimulate PI turnover in vivo in response to PDGF, and, indeed, deletion of the entire PLC$_\gamma$1 Src homology region abolishes the in vivo association of PLC$_\gamma$1 with the PDGF-R (*27*). Similarly, the *IRA* gene products, which stimulate Ras GTPase activity in yeast, contain a region related to the COOH-terminus of mammalian GAP, but do not possess SH2 domains (*28*). The ability of bacterial SH2 domains to mimic in vitro the interactions of native PLC$_\gamma$ or GAP with activated growth factor receptors provides direct evidence that these sequences are sufficient for receptor-binding. These data strongly suggest that during the evolution of cellular signaling mechanisms, the acquisition of SH2 domains conferred on PLC$_\gamma$ and GAP the capacity to interact with transmembrane tyrosine kinases, and thereby to couple growth factor stimulation to PI turnover and the Ras pathway.

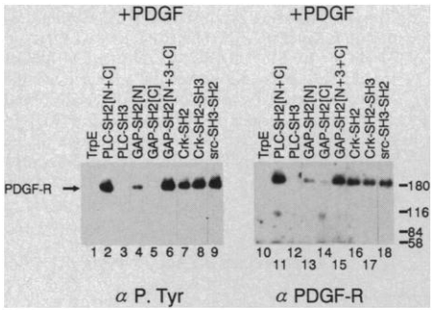


**Fig. 3.** Binding of TrpE fusion proteins that contain the GAP, Src, or Crk SH2 domains to PDGF-R in lysates of PDGF-stimulated Rat-2 cells. Serum-starved Rat-2 cells were stimulated for 5 min at 37°C with 75 nM BB-PDGF, lysed, and mixed with the indicated immobilized TrpE bacterial fusion proteins. Complexes were washed, resolved on 7.5% SDS-polyacrylamide gels and analyzed by immunoblotting with anti-P.Tyr (8 hour exposure; lanes 1 to 9) or with anti-PDGF-R (18 hour exposure; lanes 10 to 18).

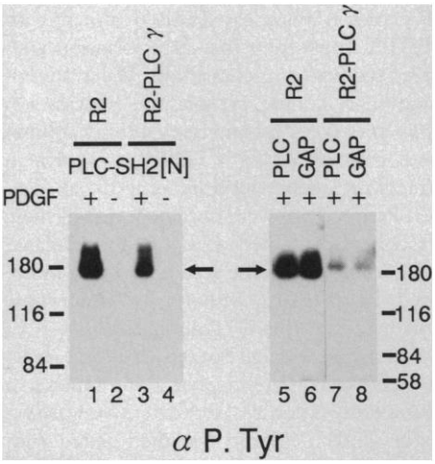


**Fig. 4.** Inhibition of in vitro binding of both PLC$_\gamma$1 and GAP SH2 domains to the activated PDGF-R in Rat-2 cells that overexpress PLC$_\gamma$1. Rat-2 cells (lanes 1, 2, 5, and 6) or a Rat-2 cell line that overexpressed PLC$_\gamma$1 by tenfold (R2-PLC$_\gamma$; lanes 3, 4, 7, 8) were stimulated with PDGF (lanes 1, 3, and 5–8) or maintained without PDGF (lanes 2 and 4). Cell lysates were mixed with immobilized TrpE-PLC-SH2[N] (lanes 1 to 4), TrpE-PLC-SH2[N + C] (lanes 5 and 7), or TrpE-GAP-SH2[N + 3 + C] (lanes 6 to 8). Samples were washed, separated by gel electrophoresis, and immunoblotted with anti-P.Tyr. Similar results were obtained by blotting with anti-PDGF-R.

The high affinity association of PLC$_\gamma$1 and GAP with the EGF-R or PDGF-R is dependent on prior growth factor stimulation, and requires receptor tyrosine kinase activity (*5, 6*). A simple explanation might be that receptor autophosphorylation elicits high affinity SH2 binding (*29*). The SH2 domains of GAP, Src, Abl, and Crk bind several proteins other than growth factor receptors (*16, 29, 30*), such as GAP-associated p62 (*18, 29*), whose common feature is tyrosine phosphorylation. In addition, autophosphorylation is required for efficient complex formation in vivo between the PDGF-R and GAP (*6*). It is feasible that tyrosine phosphorylation of residues in SH2-binding sites increases the affinity for SH2 domains. We note the presence of invariant positively charged residues in SH2, which might contribute to such an interaction (*15*). Alternatively, or in addition, growth factor stimulation and subsequent receptor autophosphorylation may induce a conformational change that forms an SH2-binding site. The affinity of PLC$_\gamma$1 and GAP for activated receptors was increased synergistically by the juxtaposition of two SH2 domains. There are several potential explanations for this synergistic effect. Growth factor-binding induces receptor dimerization, and the elevated binding activity of two linked SH2 domains may result from a cooperative interaction with the activated, dimerized receptor. Alternatively, a receptor monomer might have multiple SH2-binding sites, or the combined SH2 domains might bind more strongly to a single site. The ability of overexpressed PLC$_\gamma$1 to inhibit the in vitro binding of both PLC$_\gamma$1 and GAP SH2 domains to PDGF-R suggests that these bind to similar sites. The EGF-R phosphorylates PLC$_\gamma$1 at two tyrosines in vivo, one of which is adjacent to SH2[C] (*31*). Tyrosine phosphorylation of PLC$_\gamma$1 may decrease the affinity of SH2-binding, as the phosphorylated enzyme is apparently released from the receptor (*5*).

In summary, SH2 domains mediate the high affinity interactions of PLC$_\gamma$1 and GAP with activated growth factor receptors. The use of a specialized noncatalytic domain to direct complex formation between protein kinases and their presumptive targets is unprecedented. It is possible that a function of tyrosine phosphorylation is to regulate heteromeric protein-protein interactions.

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19. Restriction sites were introduced on either side of SH2 coding sequences in the cDNA's for bovine PLC$_\gamma$1 and human GAP with oligonucleotide-directed mutagenesis [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1987)]. For each individual SH2 domain an Sph I site was created at the 5′ end and an Nhe I site at the 3′ end. These Sph I–Nhe I fragments were cloned into a pATH bacterial *trpE* expression vector whose multiple cloning site had been modified to contain unique Sph I and Nhe I sites. For fusions that contained both SH2 domains, the Sph I site of the NH$_2$-terminal SH2 domain and the Nhe I site of the

COOH-terminal SH2 domain were used for the excision. Src and Crk fusion proteins utilized natural restriction sites. The resulting fusion proteins contained the $NH_2$-terminal 323 amino acids of TrpE and retained the desired reading frame for $PLC_\gamma 1$ or GAP.

20. Cultures of *E. coli* RR1 with pATH expression plasmids were grown, induced, and lysed as described (*29*). The TrpE fusion proteins were recovered from the supernatants by immunoprecipitation with polyclonal anti-TrpE antiserum immobilized on protein A-Sepharose beads. Immune complexes were washed (*29*), aliquoted, flash-frozen, and stored at −70°C until mixed with mammalian cell lysates. Starved or growth factor-stimulated rat fibroblasts (~$5 \times 10^6$) were lysed in 2 ml of lysis buffer (50 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Clarified mammalian cell lysate (1 ml) was mixed with immobilized bacterial fusion protein by gentle inversion for 90 min at 4°C. Complexes were recovered by centrifugation, washed three times with HNTG buffer (20 mM Hepes pH 7.0, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM $Na_3VO_4$), and analyzed by immunoblotting with anti-P.Tyr or anti-receptor as described (*6, 15, 18, 29*). To ensure that the different TrpE fusion proteins were present in similar amounts in the immune complexes incubated with the mammalian cell lysates, duplicate samples for anti-P.Tyr and anti-EGF-R immunoblotting were probed with an anti-TrpE monoclonal antibody. Equivalent amounts of the various TrpE fusion proteins were detected.
21. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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32. Bacterial fusion proteins contained the following amino acid residues: Src-SH3-SH2, $p60^{v\text{-}src}$ 87 to 291; PLC-SH2[N], bovine $PLC_\gamma 1$ 547 to 659; PLC-SH2[C], $PLC_\gamma 1$ 663 to 752; PLC-SH2[N + C], $PLC_\gamma 1$ 547 to 752; PLC ΔSH2-SH2-3, $PLC_\gamma 1$, 555 to 951; PLC-SH3, $PLC_\gamma 1$ 781 to 855; GAP-SH2[N], human GAP 178 to 277; GAP-SH2[C], GAP 348 to 444; GAP-SH2[N + 3 + C], GAP 178 to 444; Crk-SH2, $p47^{gag\text{-}crk}$ 205 to 386; Crk-SH2-SH3, $p47^{gag\text{-}crk}$ 220 to 427.
33. Equivalent contributions were made by the first two authors. We thank M. Hoffmann, J. Knopf, and G. S. Martin for critical review of the manuscript; B. Margolis and J. Schlessinger for anti-EGF-R antibodies; S. A. Courtneidge for anti-PDGF-R antibodies; K. Letwin for anti-P.Tyr antibodies; J. Knopf for bovine $PLC_\gamma 1$ cDNA; F. McCormick for human GAP cDNA; H. Hanafusa for v-*crk* DNA; J. M. Bishop for SR-A RSV v-*src* DNA; and M. Weber for R1hER cells. Supported by grants from the National Cancer Institute of Canada (NCIC) and the Medical Research Council of Canada (MRC). D.A. and M.F.M. are postdoctoral fellows of the NCIC. T.P. is a Terry Fox Cancer Research Scientist of the NCIC. C.A.K. is a graduate fellow of the MRC.

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# A Cytoplasmic Protein Inhibits the GTPase Activity of H-Ras in a Phospholipid-Dependent Manner

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**A cytoplasmic protein has been identified that inhibits the guanosine triphosphatase (GTPase) activity of bacterially synthesized, cellular H-Ras protein. This GTPase inhibiting protein is able to counteract the activity of GTPase activating protein (GAP), which has been postulated to function as a negative regulator of Ras activity. The potential biological importance of the GTPase inhibiting protein is further supported by its interaction with lipids. Phospholipids produced in cells as a consequence of mitogenic stimulation increase the activity of the GTPase inhibiting protein, as well as inhibit the activity of GAP. The interaction of such lipids with each of these two regulatory proteins would, therefore, tend to increase the biological activity of Ras and stimulate cell proliferation.**

ALTHOUGH IT APPEARS THAT CELLULAR Ras proteins are important in proliferative signal transduction (*1, 2*), the mechanism by which Ras is regulated remains unclear. As with other guanine nucleotide–binding proteins, Ras is thought to be biologically active when bound to guanosine triphosphate (GTP); its inherent GTPase activity, however, slowly converts Ras-GTP to Ras-GDP (guanosine diphosphate), which is thought to be biologically inactive. A change in either the nucleotide exchange rate of Ras or the inherent GTP hydrolysis rate could therefore be responsible for controlling the biological activity of the protein. A GTPase activating protein (GAP) has been identified (*3–5*) that stimulates the GTPase activity of normal Ras more than 100-fold without affecting oncogenic mutant Ras proteins. Because GAP can stimulate the conversion of Ras-GTP into Ras-GDP, it could function in suppressing the biological activity of Ras (*6*). On the other hand, on the basis of mutation analysis indicating that GAP interacts with a sequence that is essential for Ras biological activity (*3, 4*), it has been suggested that GAP might act as the Ras effector protein.

On the basis of results of microinjection experiments, we previously suggested that the biological activity of Ras could be controlled by phospholipids (*7, 8*). This was supported by our observation that GAP is inhibited by certain phospholipids in vitro (*8*), and we further demonstrated that GAP physically associates with lipids including phosphatidic acid (PA), phosphatidylinositol monophosphate, and arachidonic acid; other lipids were ineffective (*9*). The physical association between GAP and phospholipids resulted in the retention of GAP on a lipid affinity column. GAP bound to such a PA column was released after treatment with EDTA, suggesting that its association with lipid requires divalent cations. When a preparation of soluble, cytoplasmic proteins was passed over a PA-containing affinity column and then eluted with EDTA, two protein fractions with opposite effect on Ras GTPase activity were obtained: one of these fractions contained GAP and stimulated GTPase, whereas the second fraction inhibited Ras GTPase activity. We have now characterized this GTPase inhibitor.

A mouse brain extract that had been eluted from DEAE-Sephacel was added to a PA affinity column. After extensive washing (Fig. 1), the column was then eluted with 10 mM EDTA. The GTPase activity of H-Ras was inhibited by eluted fractions 1 to 3 (*10*) (Fig. 1A). Later fractions eluted from the same column contained GAP (*9*). Thus, there were two distinct enzymatic activities in mouse brain extract that bind to PA in a cation-dependent manner. One is GAP, whereas the other is a GTPase inhibiting protein. We confirmed these results, which were observed with a filter binding assay (*11*) (Fig. 1A), by immunoprecipitation analysis (Fig. 1B) (*12*).

In the immunoprecipitation assay, an in-

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