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G protein $\beta\gamma$ subunits stimulate phosphorylation of Shc adapter protein

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ABSTRACT **The mechanism of mitogen-activated protein (MAP) kinase activation by pertussis toxin-sensitive G_i-coupled receptors is known to involve the $\beta\gamma$ subunits of heterotrimeric G proteins ($G\beta\gamma$), $p21^{ras}$ activation, and an as-yet-unidentified tyrosine kinase. To investigate the mechanism of $G\beta\gamma$-stimulated $p21^{ras}$ activation, $G\beta\gamma$-mediated tyrosine phosphorylation was examined by overexpressing $G\beta\gamma$ or α2-C10 adrenergic receptors (ARs) that couple to G_i in COS-7 cells. Immunoprecipitation of phosphotyrosine-containing proteins revealed a 2- to 3-fold increase in the phosphorylation of two proteins of ≈50 kDa (designated as p52) in $G\beta\gamma$-transfected cells or in α2-C10 AR-transfected cells stimulated with the agonist UK-14304. The latter response was pertussis toxin sensitive. These proteins (p52) were also specifically immunoprecipitated with anti-Shc antibodies and comigrated with two Shc proteins, 46 and 52 kDa. The $G\beta\gamma$- or α2-C10 AR-stimulated p52 (Shc) phosphorylation was inhibited by coexpression of the carboxyl terminus of β-adrenergic receptor kinase (a $G\beta\gamma$-binding pleckstrin homology domain peptide) or by the tyrosine kinase inhibitors genistein and herbimycin A, but not by a dominant negative mutant of $p21^{ras}$. Wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K) inhibited phosphorylation of p52 (Shc), implying involvement of PI3K. These results suggest that $G\beta\gamma$-stimulated Shc phosphorylation represents an early step in the pathway leading to $p21^{ras}$ activation, similar to the mechanism utilized by growth factor tyrosine kinase receptors.**

Many extracellular signals leading to cell growth and differentiation are transmitted by two major classes of cell surface receptors, tyrosine kinase growth factor receptors and G protein-coupled receptors (1). The mechanism of tyrosine kinase receptor-stimulated mitogenic signaling involves a series of protein–protein interactions mediated by src homology (SH) 2 and SH3 domains that bind to phosphotyrosine residues and proline-rich peptides, respectively (2). These protein–protein interactions serve to localize signaling intermediates to appropriate membrane compartments and lead to activation of $p21^{ras}$/mitogen-activated protein (MAP) kinase. While the G protein-coupled receptor signaling pathway has been thought to be totally independent of the tyrosine kinase receptor pathway, recent evidence has demonstrated that G protein-coupled receptors utilize the same effectors, such as $p21^{ras}$ and MAP kinase, in mitogenic signaling pathways (1, 3).

G protein-coupled receptors such as α-adrenergic receptors (ARs), muscarinic acetylcholine receptors, dopamine receptors, and those for lysophosphatidic acid (LPA), thrombin, bombesin, or endothelin have all been shown to promote activation of MAP kinase. G_q-coupled receptors generally initiate a $p21^{ras}$-independent pathway involving protein kinase C (PKC), whereas the pertussis toxin (PTX)-sensitive G_i-coupled receptors utilize a pathway that requires $p21^{ras}$ activation in a PKC-independent manner (1, 4, 5). Recent evidence has demonstrated that the mechanism of $p21^{ras}$/MAP kinase activation by G_i-coupled receptors involves the $\beta\gamma$ subunits of heterotrimeric G proteins ($G\beta\gamma$). In COS-7 cells, this pathway is attenuated by coexpression of the α subunit of the G protein transducin ($G_t\alpha$), acting to sequester $G\beta\gamma$ (6). Further, direct overexpression of $G\beta\gamma$ subunits results in activation of MAP kinase through $p21^{ras}$ (5, 7). In Rat-1 cell lines, expression of a $G\beta\gamma$ antagonist derived from the β-adrenergic receptor kinase (βARK) carboxyl-terminal region (βARKct) inhibits G_i-linked LPA-stimulated $p21^{ras}$ activation, further implicating the involvement of $G\beta\gamma$ in this pathway (8). Moreover, the $p21^{ras}$/MAP kinase pathway mediated by G_i-coupled receptors is inhibited by the tyrosine kinase inhibitors genistein and herbimycin A, suggesting that a tyrosine phosphorylation step is also involved in the pathway (4, 5).

In this study, we examine the potential site of $G\beta\gamma$-stimulated tyrosine phosphorylation in the pathway leading from G_i-coupled receptors to $p21^{ras}$ activation. The results strongly implicate Shc, an adapter protein known to be involved in tyrosine kinase receptor-mediated mitogenic signaling, as the locus of this phosphorylation.

MATERIALS AND METHODS

Materials. COS-7 cells were from the American Type Culture Collection; culture media and LipofectAMINE, from GIBCO/BRL; fetal bovine serum (FBS) and gentamicin, from Life Technologies (Gaithersburg, MD); staphylococcal protein A- or G-plus-agarose, goat anti-rabbit IgG and goat anti-mouse IgG, from Oncogene Science; herbimycin A, genistein, and phorbol 12-myristate 13-acetate (PMA), from Sigma; UK-14304, from Pfizer; PTX, from List Biologicals (Campbell, CA); monoclonal anti-phosphotyrosine antibody (PY20) and rabbit polyclonal anti-Shc antibody, from Transduction Laboratories (Lexington, KY); silica gel TLC plate, from Kodak; phosphoamino acid standards and wortmannin, from Sigma; [^{32}P]orthophosphate and enhanced chemiluminescence (ECL) kit, from Amersham; and Immobilon-P, from Millipore. The cDNAs for the human α2-C10 AR and βARK1 were cloned in our laboratory. The cDNAs encoding Gβ2 and Gγ2 were provided by M. Simon (California Institute of Technology), and DNA encoding the $p21^{N17ras}$ dominant-negative mutant was from D. Altschuler and M. Ostrowski (Duke University). The βARKct-encoding minigene [containing a pleckstrin homology (PH) domain] was prepared as described (9).

Cell Culture and Transfection. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) in 6-well

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Abbreviations: SH, src homology; MAP, mitogen-activated protein; AR, adrenergic receptor; PKC, protein kinase C; PTX, pertussis toxin; $G\beta\gamma$, $\beta\gamma$ subunits of heterotrimeric G protein; PMA, phorbol 12-myristate 13-acetate; PH domain, pleckstrin homology domain; βARK, β-adrenergic receptor kinase; βARKct, carboxyl-terminal region of βARK; FBS, fetal bovine serum; PI3K, phosphatidylinositol 3-kinase.

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culture plates and were transfected by using LipofectAMINE as described (10). After transfection, cells were incubated at 37°C in DMEM containing 10% FBS overnight. Cells were then incubated overnight in DMEM containing 0.5% FBS prior to experiments. Concentrations of UK-14304, genistein, PMA, herbimycin A, wortmannin, and PTX are indicated in figure legends.

^{32}P Labeling and Immunoprecipitation. Three hours prior to the assay, the medium was replaced by phosphate-free DMEM containing [^{32}P]orthophosphate at 200 μCi/ml (1 μCi = kBq). Appropriate agonists were directly added to the medium, and after the indicated times, the cells were chilled and washed with ice-cold phosphate-buffered saline (PBS) (GIBCO/BRL). Cells were subsequently scraped in 0.8 ml of ice-cold lysis buffer [30 mM Tris/150 mM NaCl/10 mM EDTA/0.5% sodium deoxycholate/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/protease-inhibitor cocktail (ICN)/1 mM sodium vanadate, pH 7.4]. After 20 min, the lysate was centrifuged for 20 min and precleared by incubation for 30 min with protein G-plus- or protein A-agarose beads coated with mouse or rabbit total IgG. The precleared lysates were incubated with anti-phosphotyrosine antibody PY20 (4 μg) (1 h, 4°C) followed by protein G-plus-agarose (30 min), or with anti-Shc antibody (2 μg) followed by protein A-agarose. The immunoprecipitates were washed four times with lysis buffer, and protein complexes were eluted in SDS/sample buffer and were analyzed by SDS/PAGE (4–20% gradient, NOVEX; San Diego). Gels were dried and exposed to Kodak Biomax films. The bands of interest were quantitated by PhosphorImager analysis (Molecular Dynamics).

Phosphoamino Acid Analysis. ^{32}P-labeled proteins separated by SDS/PAGE were transferred onto Immobilon-P by electroblotting and exposed to film. The desired labeled bands were excised and the strips of membrane were rinsed several times with water. Each strip was incubated in 200 μl of 6 M HCl for 1 h at 110°C. The supernatant was collected and evaporated by Speed-Vac (Savant), and the sample was dissolved in 10 μl of pH 1.9 buffer (7.8% acetic acid/2.5% formic acid, vol/vol in water). Two-dimensional electrophoresis of phosphoamino acids was performed on TLC plates (Kodak) as described, using pH 1.9 buffer for the first dimension and pH 3.5 buffer (5% acetic acid/0.5% pyridine, vol/vol in water) for the second dimension. The TLC plate was exposed to the x-ray film for 1–2 months.

RESULTS AND DISCUSSION

G_i-coupled receptor-mediated p21^{ras}/MAP kinase activation appears to involve a tyrosine kinase (4, 5) and to be initiated by the release of G$\beta\gamma$ subunits (6–8). To determine which proteins show an increase in tyrosine phosphorylation upon G_i stimulation, G$\beta\gamma$-transfected or α2-C10 AR-transfected COS-7 cells were labeled with $^{32}P_i$ and phosphotyrosine-containing proteins were immunoprecipitated by anti-phosphotyrosine antibodies. As shown in Fig. 1*A*, an increase in phosphorylation of two proteins of ≈50 kDa (designated collectively as p52) was observed in G$\beta\gamma$-overexpressing cells. PhosphorImager analysis of the two phosphorylated bands demonstrated that both bands were equally increased in intensity (i.e., 2- to 3-fold) in G$\beta\gamma$-transfected cells (Fig. 1*B*). An increase in tyrosine phosphorylation of p52 was confirmed as determined by phosphoamino acid analyses, although an increase of serine phosphorylation was also observed (data not shown). p52 phosphorylation in G$\beta\gamma$-overexpressing cells was attenuated by genistein, a general tyrosine kinase inhibitor, as well as by herbimycin A, a src-family kinase-specific inhibitor (Fig. 1*B*). The inhibitory effect of genistein was more marked than that of herbimycin A, a finding which parallels their effects on MAP kinase activation via α2-C10 AR stimulation (5).

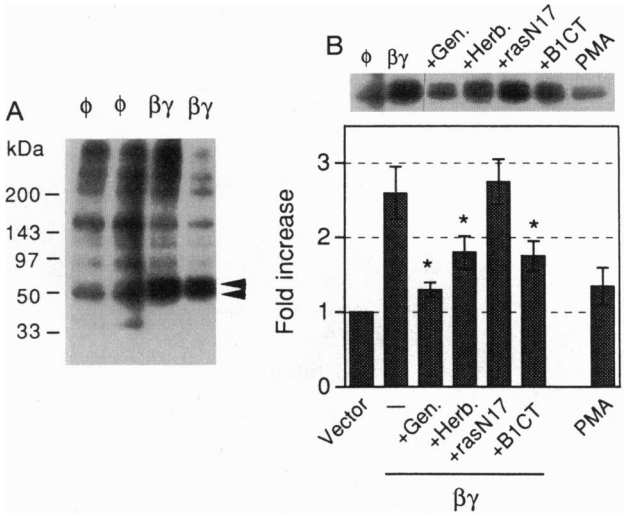

FIG. 1. Stimulation of p52 phosphorylation in G$\beta\gamma$-overexpressing cells. (*A*) COS-7 cells, transiently transfected with Gβ2 and Gγ2 subunits (1 μg of DNA each per well), were labeled with [^{32}P]-orthophosphate for 3 h. Phosphotyrosine-containing proteins were immunoprecipitated from cell lysates by using anti-phosphotyrosine antibody PY20, and the samples were analyzed on SDS/polyacrylamide gels (4–20% gradient gel). Arrowheads indicate phosphotyrosine-containing proteins with increased phosphorylation in G$\beta\gamma$-transfected cells (designated as p52) on the autoradiogram. Shown is a representative autoradiogram. ϕ, Empty plasmid vector-transfected cells (control); $\beta\gamma$, Gβ2- and Gγ2-transfected cells. (*B*) Effects of various reagents on p52 phosphorylation in G$\beta\gamma$-transfected cells—i.e., cotransfection of a dominant negative mutant of p21^{ras} (rasN17) or βARKct minigene (B1CT), or tyrosine kinase inhibitors (genistein or herbimycin A). Prior to the labeling, cells were treated with 100 μM genistein or herbimycin A at 1.0 μg/ml for 10 min, and the inhibitor was also included in the ^{32}P-labeling step at the same concentrations. Treatment of untransfected COS-7 cells with phorbol ester, PMA (1 μM for 5 min), which had been incubated in serum-free medium, did not induce p52 phosphorylation. (*Upper*) Autoradiogram of p52 phosphorylation. (*Lower*) Quantitation of the bands by PhosphorImager analysis. The autoradiogram is from a representative experiment, and data shown represent means ± SEM values for four to six separate experiments performed in duplicate. *, Value less than G$\beta\gamma$-stimulated, $P < 0.05$. Vector or ϕ, empty plasmid vector-transfected cells; —, Gβ- and Gγ-transfected cells; +Gen., genistein-treated G$\beta\gamma$-transfected cells; +Herb., herbimycin A-treated G$\beta\gamma$-transfected cells; +rasN17, p21^{N17ras}-cotransfected G$\beta\gamma$-transfected cells; +B1CT, βARKct minigene-cotransfected G$\beta\gamma$-transfected cells; PMA, PMA-stimulated cells.

We have previously used several PH domain peptides which bind G$\beta\gamma$ to inhibit various G$\beta\gamma$-mediated processes such as inositol phosphate production or p21^{ras}/MAP kinase activation (8–10). Similarly, p52 phosphorylation was also attenuated by coexpression of the G$\beta\gamma$-sequestering βARKct peptide (which contains a PH domain), suggesting that the increase in phosphorylation is mediated by the overexpressed G$\beta\gamma$ subunits (Fig. 1*B*). In contrast, coexpression of a dominant negative mutant of p21^{ras} (rasN17) did not affect p52 phosphorylation, indicating that the relevant phosphorylation step is not downstream of p21^{ras} (Fig. 1*B*). Since G$\beta\gamma$ also stimulates phosphatidylinositol hydrolysis, resulting in PKC activation, we assessed whether G$\beta\gamma$-stimulated p52 phosphorylation was PKC dependent. Exposure to phorbol ester (PMA), a PKC activator, however, did not provoke p52 phosphorylation (Fig. 1*B*). Thus, PKC activation does not lead to the stimulation of a tyrosine kinase which would result in the increased phosphorylation of p52.

Stimulation of the α2-C10 AR G_i-coupled receptor also induced p52 phosphorylation in a PTX-sensitive manner. Basal tyrosine phosphorylation of p52 in PTX-treated cells was higher than that in untreated cells (Fig. 2). Maximal p52

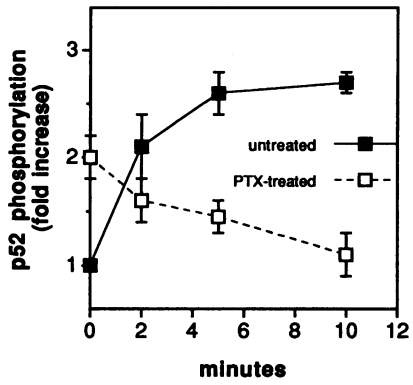

FIG. 2. Kinetics of α2-C10 AR-mediated increase of p52 phosphorylation. [^{32}P]Orthophosphate-labeled α2-C10 AR-transfected COS-7 cells were stimulated with agonist, UK-14304 (1 μM), for the indicated periods of time. Phosphotyrosine-containing proteins were immunoprecipitated and analyzed. The same set of cells were incubated overnight in serum-free medium with PTX at 100 ng/ml. The p52 on the autoradiogram was quantitated by PhosphorImager analysis, and the relative intensities are presented as fold increase over basal. Data shown represent means ± range for two separate experiments performed in duplicate.

phosphorylation was observed at 5 min after agonist stimulation. Together with the results from Gβγ-transfected cells (Fig. 1), these results are consistent with the idea that tyrosine phosphorylation of p52 may be involved in the Gβγ-mediated pathway leading to p21^{ras} activation.

Several antibodies were utilized in attempts to immunoprecipitate p52. Among them, only anti-Shc antibodies could immunoprecipitate p52 from Gβγ-overexpressing COS-7 cells, which comigrated with two Shc proteins, 46 and 52 kDa (Fig. 3 *A* and *B*). The increase in anti-Shc-immunoprecipitated p52 in Gβγ-transfected cells (i.e., 2.5-fold) was similar to that in anti-phosphotyrosine-immunoprecipitated p52 (Fig. 1*B*). A

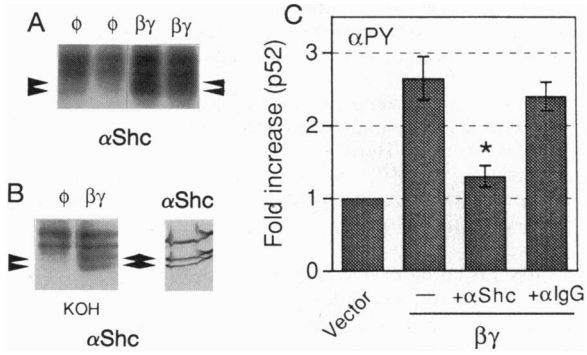

FIG. 3. Increase of p52 phosphorylation is due to an increase in Shc phosphorylation. (*A*) Anti-Shc antibody (αShc) immunoprecipitates p52 with 2.5-fold-increased phosphorylation from Gβγ-transfected cells. Arrowheads indicate the position of p52 on the autoradiogram. ϕ, Empty plasmid vector-transfected cells; βγ, Gβ- and Gγ-transfected cells. (*B*) Anti-Shc antibody immunoprecipitates p52 with increased tyrosine phosphorylation from Gβγ-transfected cells (i.e., 90% ± 10% increase). (*Left*) Autoradiogram of the gel that has been treated with 1 M KOH. (*Right*) Shc (46, 52, and 66 kDa) in COS-7 cells as visualized on the anti-Shc immunoblot by using an ECL kit. (*C*) Prior to the anti-phosphotyrosine (αPY) antibody immunoprecipitation, ^{32}P-labeled Gβγ-transfected cell lysates were treated with anti-Shc antibody to preclear the proteins recognized by the anti-Shc antibody (+αShc). As a control, goat anti-rabbit total IgG was utilized (+αIgG). The preclearing with anti-Shc antibody results in the loss of anti-phosphotyrosine-immunoprecipitated p52. The relative intensities of anti-phosphotyrosine-immunoprecipitated p52 are presented as fold increase over basal. Data are the mean ± SEM of three separate experiments performed in duplicate. *, Value less than Gβγ-stimulated, $P < 0.05$.

90% increase in phosphorylation of the anti-Shc-immunoprecipitated p52 was still detected after treatment of gels with 1 M KOH (which hydrolyzes phosphoserine and -threonine preferably to phosphotyrosine), confirming the increase of tyrosine phosphorylation of p52 Shc (Fig. 3*B*). Phosphoamino acid analysis of anti-Shc-immunoprecipitated p52 revealed a similar increase (i.e., 100% increase) in phosphotyrosine in Gβγ-transfected cells (data not shown). Moreover, "preclearing" the COS-7 cell extracts with anti-Shc antiserum resulted in the loss of p52 from anti-phosphotyrosine antibody immunoprecipitates (i.e., 85% decrease of Gβγ-stimulated p52 phosphorylation), confirming that p52 is recognized by anti-Shc antibodies (Fig. 3*C*). These results strongly suggest that the p52 is Shc and that the increase of p52 phosphorylation in Gβγ-overexpressing cells represents Shc phosphorylation.

Shc is an adapter protein that is both serine- and tyrosine-phosphorylated upon activation of tyrosine kinase receptors such as the epidermal growth factor receptor or the insulin receptor (11–13). Tyrosine phosphorylated Shc associates with the Grb2/SOS complex through the SH2 domain of Grb2, thereby leading to p21^{ras} activation. Two Shc proteins, 46- and 52-kDa, are derived from the same cDNA, while the 66-kDa Shc protein is encoded by a distinct transcript (11). In this study, an increase in tyrosine phosphorylation was observed on both 46- and 52-kDa proteins visualized as an apparent doublet.

It has also been reported that tyrosine phosphorylation of Shc is induced upon stimulation of some G protein-coupled receptors such as those for endothelin (14) and thyrotropin-releasing hormone (15). Although these receptors are thought to couple to PTX-insensitive G proteins, perhaps through $G\alpha_q$ or $G\alpha_{11}$, it remains possible that tyrosine phosphorylation of Shc in these cases is also modulated by Gβγ released upon receptor activation. Since tyrosine phosphorylation of Shc after activation of tyrosine kinase receptors results in activation of p21^{ras} and MAP kinase by means of a Shc/Grb2/SOS complex (12, 13), it is tempting to speculate that this same complex is involved in Gβγ-mediated p21^{ras} and MAP kinase activation.

Tyrosine phosphorylation of Shc is likely associated with activation of a protein tyrosine kinase. Some G protein-coupled receptors such as the receptor for thrombin are known to stimulate pp60^{src} activity (16). Since an increase in tyrosine phosphorylation of Shc has been observed in pp60$^{v\text{-}src}$-transformed cells (17), it is possible that pp60^{src} is activated by a mechanism involving Gβγ subunits. However, there has been no precedent for direct pp60^{src} activation by Gβγ. pp60^{src} activity is regulated by c-src kinase and phosphotyrosine phosphatase (18). In this regard, an intriguing observation is that phosphotyrosine phosphatase PTP1D is shown to be essential in thrombin-mediated fibroblast proliferation (19). Moreover, phosphorylation and stimulation of SH-PTP is shown to be coupled to platelet thrombin receptor via a PTX-sensitive G protein (20). Thus, phosphotyrosine phosphatase may link the tyrosine kinase activity to Gβγ. Alternatively, a PH domain-containing tyrosine kinase might be directly activated by Gβγ by a translocation mechanism in a way similar to that of βARK.

Another possible Gβγ effector in this pathway, phosphatidylinositol 3-kinase (PI3K), has been shown to be involved in both growth factor receptor- and G protein-coupled receptor-mediated signaling (21). Gβγ-sensitive PI3K has been reported in neutrophils and platelets (22, 23). We assessed whether PI3K might be involved in the pathway leading to Shc phosphorylation by using wortmannin, a specific inhibitor of PI3K activity. The phosphorylation of p52 Shc is potently inhibited by wortmannin at $IC_{50} \approx 20$ nM; maximal inhibition was ≈70% (Fig. 4). In contrast, epidermal growth factor (EGF)-stimulated Shc phosphorylation by means of direct tyrosine phosphorylation by the EGF receptor is not inhibited

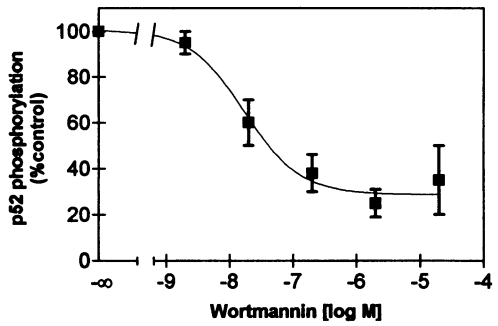

FIG. 4. Dose–response curve of wortmannin inhibition of Gβγ-induced phosphorylation of p52 Shc. Prior to the labeling, cells were treated with the indicated concentrations of wortmannin for 10 min, and wortmannin was also included in the ^{32}P-labeling step at the same concentrations. Phosphotyrosine-containing proteins were immunoprecipitated and analyzed. The p52 Shc on the autoradiogram was quantitated by PhosphorImager analysis, and the relative intensities are presented as percentage of Gβγ-induced increase of p52 Shc phosphorylation. Data are the mean ± SEM of three separate experiments.

by 20 nM wortmannin (data not shown). It is of note that PI3K is known to associate with src-family tyrosine kinases through the SH2 and SH3 domains of src-family kinases (24, 25). Therefore, it is possible that irreversible binding of wortmannin to the catalytic subunit of PI3K (26) may affect the src-family kinase activity. Alternatively, PI3K may be involved in this Gβγ-mediated pathway at a point upstream of the tyrosine kinase.

On the basis of evidence that G_i-coupled receptor-mediated p21^{ras}/MAP kinase activation is attenuated by Gβγ-binding PH domain peptides (10), it is tempting to speculate that the direct Gβγ effector may possess a PH domain, and it is recruited to the plasma membrane, where the tyrosine phosphorylation is triggered. It remains to be determined how Gβγ leads to activation of a tyrosine kinase and tyrosine phosphorylation of Shc. Nonetheless, the demonstration of Gβγ-mediated Shc phosphorylation and the effect of wortmannin suggests that the G protein-coupled receptor-mediated mitogenic pathway converges at an early point with the tyrosine kinase receptor pathway (Fig. 5) (27).

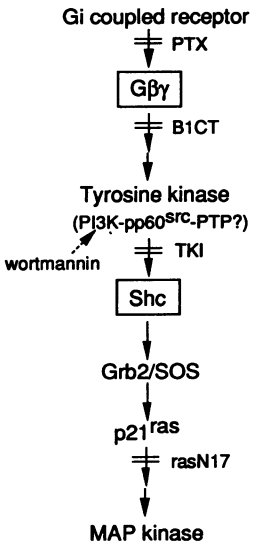

FIG. 5. Proposed signaling pathway of p21^{ras}/MAP kinase activation triggered by G_i-coupled receptor via Gβγ subunits. The release of Gβγ subunits from PTX-sensitive G protein-coupled receptors initiates reactions leading to activation of a tyrosine kinase (possibly pp60^{src} kinase) through an unknown mechanism, resulting in Shc tyrosine phosphorylation. PI3K is one of the candidates involved at a site between Gβγ and the tyrosine kinase. Involvement of protein tyrosine phosphatase (PTP) has also been proposed. The pathway leading to p21^{ras} activation through the SH2 and SH3 domain interaction of Grb2 and SOS is well established (2). PTX specifically blocks the release of Gβγ due to G_i-coupled receptor stimulation. βARKct peptide (B1CT), containing a PH domain, specifically sequesters Gβγ. TKI, tyrosine kinase inhibitor.

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