Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8258–8263, August 1996
Biochemistry

Transformation of Rat-1 fibroblasts with the v-*src* oncogene increases the tyrosine phosphorylation state and activity of the α subunit of Gq/G11

(signal transduction/guanine nucleotide-binding proteins/oncogenes/tyrosine kinase/endothelin)

WENDY W. LIU, RAYMOND R. MATTINGLY*, AND JAMES C. GARRISON†

Department of Pharmacology, School of Medicine, University of Virginia, Charlottesville, VA 22908

Communicated by Oscar L. Miller, Jr., University of Virginia, Charlottesville, VA, March 8, 1996 (received for review January 4, 1996)

ABSTRACT **Two major intermediaries in signal transduction pathways are pp60$^{v\text{-}src}$ family tyrosine kinases and heterotrimeric guanine nucleotide-binding proteins. In Rat-1 fibroblasts transformed by the v-*src* oncogene, endothelin-1 (ET-1)-induced inositol 1,4,5-trisphosphate accumulation is increased 6-fold, without any increases in the numbers of ET-1 receptors or in the response to another agonist, thrombin. This ET-1 hyperresponse can be inhibited by an antibody directed against the carboxyl terminus of the Gq/G11 α subunit, suggesting that the Gq/G11 protein couples ET-1 receptors to phospholipase C (PLC). While v-*src* transformation did not increase the expression of the Gq/G11 α subunit, immunoblotting with anti-phosphotyrosine antibodies and phosphoamino acid analysis demonstrated that the Gq/G11 α subunit becomes phosphorylated on tyrosine residues in v-*src*-transformed cells. Moreover, when the Gq/G11 protein was extracted from control and transformed cell lines and reconstituted with exogenous PLC, AlF_4^--stimulated Gq/G11 activity was markedly increased in extracts from v-*src*-transformed cells. Our results demonstrate that the process of v-*src* transformation can increase the tyrosine phosphorylation state of the Gq/G11 α-subunit in intact cells and that the process causes an increase in the Gq/G11 α-subunit's ability to stimulate PLC following activation with AlF_4^-.**

It is widely held that protooncogenes encode proteins that are involved in the normal pathways of growth control and that oncogene products induce constitutive proliferation through alteration of these pathways (1). The product of the v-*src* gene, pp60$^{v\text{-}src}$, is a nonreceptor tyrosine kinase, and its expression leads to morphological transformation and aberrant proliferation. pp60$^{v\text{-}src}$ has been demonstrated to alter the pathways of phosphatidylinositol metabolism in a number of cell types (2, 3), although the biochemical mechanisms involved have not been clearly defined. Our previous work with Rat-1 fibroblasts transformed with the v-*src* oncogene has demonstrated these cells to be a useful model system for understanding the interactions between pp60$^{v\text{-}src}$ and the pathways of phosphatidylinositol metabolism (4–6). This cell line contains both endothelin-1 (ET-1) and thrombin receptors coupled to the phospholipases capable of generating inositol phosphates. In the normal Rat-1 cell, both ET-1 and thrombin are able to induce a transient, 4- to 6-fold increase in inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] levels. However, in v-*src*-transformed cells, but not in cells transformed by *ras*, the response to ET-1 is markedly increased while the response to thrombin is not (4–6). We have demonstrated that the hyperresponse to ET-1 can be observed in plasma membrane preparations and that there is no apparent change in the number of ET-1 receptors in the membranes (6). The focus of the present work was to determine why the effects of v-*src* transformation were limited to signals generated by the ET-1 receptor and to elucidate the molecular mechanism by which the pp60$^{v\text{-}src}$ tyrosine kinase affects the signaling system coupled to this receptor. We have discovered that the α subunit of the guanine nucleotide-binding protein(s) [G protein(s)] Gq and/or G11 is phosphorylated on tyrosine residues and is more active in *in vitro* assays measuring its ability to activate the β isoform of phospholipase C (PLC-β).

MATERIALS AND METHODS

Cell Culture. Transformed Rat-1 fibroblasts (Rat-1 v-*src*) and their nontransformed counterparts (Rat-1 *neo*) were grown in 100-mm culture dishes in DMEM containing 10% fetal bovine serum as described (4–6). For experiments using herbimycin A, cells were incubated in serum-free DMEM for 12 hr, either with 0.5 μg of herbimycin A per ml diluted from stock in dimethyl sulfoxide or with dimethyl sulfoxide only as a control.

Agonist-Stimulated Inositol Phosphate Production. Cells approaching confluence were labeled with 5 μCi of [^{3}H]inositol for 24 hr in serum-free medium, and 100 mM LiCl was added for the last 30 min as described (5, 6). The intact cells were stimulated with either ET-1 or thrombin for 5 min as described (5, 6). Acid extracts were prepared and [^{3}H]inositol phosphates were measured after separation by HPLC (5). For experiments in which pertussis toxin (PTX) was used, cells were incubated with the toxin for the final 2 hr of the 24-hr labeling period (7).

[^{3}H]Inositol Phosphate Release from Labeled Membranes. The Rat-1 cells were labeled with [^{3}H]inositol and the plasma membranes were isolated as described (6). Aliquots of reaction buffer, together with 1 mM GTP, agonists, and 100 nM $CaCl_2$, were warmed to 37°C for 5 min, and then the reaction was initiated by the addition of the ^{3}H-labeled membranes (6). The reaction was terminated as described (6); phytate was added to 1 mg/ml final concentration, and the samples further processed for HPLC analysis as described (5). To determine whether the Gq-directed antibody QL-142 would affect phospholipase C (PLC) activation, high specific activity [^{3}H]inositol-labeled membranes at 1 mg of protein per ml were preincubated on ice for 3 hr with 100 μg of affinity-purified QL-142 per ml (8) before dilution into the PLC assay. To investigate the specificity of the antibody effects, QL-142 was preincu-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: G proteins, guanine nucleotide-binding proteins; ET-1, endothelin-1; InsP_3, inositol trisphosphate; PLC, phospholipase C; PLC-β, the β isoform of PLC; PTX, pertussis toxin; PtdInsP_2, phosphatidylinositol 1,4-biphosphate.

*Present address: Department of Pathology, University of Vermont, Burlington, VT 05405.

†To whom reprint requests should be addressed at: Department of Pharmacology, Health Sciences Center, University of Virginia, Charlottesville, VA 22908. e-mail: jcg8w@virginia.edu.

bated for 30 min on ice with 50 μM C-terminal Gq peptide (9) before addition to the membranes.

Immunoprecipitation of the Gq/G11 α Subunit. Confluent monolayers of cells were serum-starved for 24 hr and extracted for 30 min on ice with RIPA buffer [150 mM NaCl/50 mM Tris, pH 8.0/1 mM EDTA/1.0% (vol/vol) Nonidet P-40/0.5% (wt/vol) sodium deoxycholate/0.1% (wt/vol) SDS/2 μg of aprotinin per ml/500 μg of bacitracin per ml/0.4 mM phenylmethylsulfonyl fluoride/1 mM $NaVO_4$]. Lysate (1.5 mg) was incubated for 1 hr at 4°C with 5 μg of either the QL anti-Gq/11 antiserum (Santa Cruz Biotechnology) or control rabbit anti-mouse antiserum. Protein A-Sepharose [30 μl of a 50% (wt/vol) solution; Pharmacia] was added and the incubation was continued for another hour. The immunoprecipites were resolved by SDS/PAGE, transferred to nitrocellulose, and analyzed by immunoblotting using either the QL antiserum or monoclonal antiphosphotyrosine antibody (Upstate Biotechnology).

***In Vivo* Labeling of Gq/G11.** Rat-1 *neo* and Rat-1 v-*src* cells were labeled for 10 hr with 3 ml of phosphate-free DMEM per plate, containing 1 mCi of [^{32}P]PO_4 per ml and proteins extracted with RIPA buffer. The Gq/G11 in the extracts was immunoprecipitated as described above. The proteins in the immunoprecipitates were resolved on a SDS/12% polyacrylamide gel, dried, and exposed to Kodak XAR-5 film. The ^{32}P-labeled band corresponding to Gq/G11 α subunit and a closely migrating protein band were cut from the gel, rehydrated, resolved on a second SDS/polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) membrane (Schleicher & Schuell). After exposing the membrane to Kodak XAR-5 film, the ^{32}P-labeled Gq/G11 α band and the faster migrating protein were cut from the membrane for phosphoamino acid analysis.

Phosphoamino Acid Analysis. The ^{32}P-labeled Gq/G11 α and the comigrating protein on PVDF membrane were digested with 6 M HCl for 1 hr at 110°C (10) and resuspended in pH 2.5 electrophoresis buffer [5.9% (vol/vol) glacial acetic acid, 0.8% (vol/vol) formic acid, 0.3% (vol/vol) pyridine, and 0.3 mM EDTA] (11). The phosphoamino acids were resolved by one-dimensional thin layer electrophoresis on a cellulose-coated thin layer plate for 1 hr at 1000 V (10, 11). The plate was exposed to Kodak XAR-5 film for ^{32}P detection or sprayed with ninhydrin to visualize the standards.

Assay of Gq/G11-Stimulated PLC. PLC activity was determined following reconstitution into phospholipid vesicles containing the G protein α subunits as described (12). Phosphatidylinositol 1,4-biphosphate (PtdInsP_2; Calbiochem), phosphatidylethanolamine, and phosphatidylserine (Avanti Polar Lipids) with [^{3}H]PtdInsP_2 as substrate (about 10,000 cpm/assay) were combined, dried under stream of nitrogen, and redissolved in cyclohexane. The sample was frozen and lyophilized for 6 hr to overnight before resuspension in buffer A containing 15 mM Hepes (pH 7.4), 100 mM NaCl, 0.5 mM $MgCl_2$, 1 mM DTT, and 200 μM $NaVO_4$, and 0.8% sodium cholate. The lipids were sonicated at level 2 for 1 min with a 550 Sonic Dismembrator (Fisher Scientific) and combined with equal amounts of RIPA-extracted proteins (made as described above) from control or v-*src* Rat-1 cells. The mixtures were dialyzed overnight against buffer B (same as buffer A without cholate) to remove sodium cholate and other detergents and to let large unilamellar vesicles form and incorporate G proteins slowly. Samples were diluted 1.5 times after dialysis. Each assay tube contained 50 μl of lipid vesicles and 25 μl of assay buffer to give a final concentration of 37.5 mM Hepes (pH 7.0), 125 mM NaCl, 2.25 mM $MgCl_2$, 2 mM EGTA, 1-3 μM free Ca^{2+}, and 0.5 mg of BSA per ml. The reaction was started by addition of 10 ng of purified turkey erythrocyte PLC-β in the presence or absence of AlF_4^- (10 mM NaF/20 μM $AlCl_3$) and allowed to proceed for 15 min at 30°C. The reaction was stopped by addition of $CHCl_3/CH_3OH/HCl$ as described (12). The amount of [^{3}H]InsP_3 released into the aqueous phase was quantitated by scintillation counting.

RESULTS

Endothelin Receptors Are Coupled to Gq/G11. In many cells, ET-1 receptors are thought to couple to PLC-β via the Gq α subunits (13), whereas thrombin receptors are thought to couple via the Gi α subunit (14). Thus, one possible explanation for the difference in the effect of v-*src* on the ET-1 and thrombin responses in Rat-1 cells is that v-*src* only affects one of these pathways. To examine this possibility, we pretreated cells with 100 ng of PTX per ml, which blocks signal transduction by ADP-ribosylating the α subunits of the Gi and Go proteins and uncouples their associated receptors. In the experiments presented in Fig. 1, both control and v-*src*-transformed Rat-1 fibroblasts were labeled with [^{3}H]inositol and stimulated with ET-1 or thrombin, and the resulting [^{3}H]InsP_3 levels were quantitated after HPLC separation (5, 6). The data in Fig. 1*A* demonstrate the marked increase in ET-1 response produced in the v-*src* cells and show that this increase was not significantly affected by PTX. In contrast, PTX significantly reduced thrombin-stimulated InsP_3 accumulation in an analogous experiment (Fig. 1*B*). These results support the concept that the ET-1 and thrombin receptors couple to different G proteins in Rat-1 cells.

Since the levels of inositol phosphates measured in intact cells potentially reflect alterations in either their production or metabolism (5), we prelabeled both types of Rat-1 cells with [^{3}H]inositol, isolated the labeled membranes, and assayed agonist-induced release of [^{3}H]InsP_3. This protocol allowed us to localize the effects of the agonists to the level of the membrane and to probe the potential Gq/G11 interaction with the ET-1 receptor using an antibody directed to the Gq/G11 α subunit (Fig. 1 *C* and *D*). The data in Fig. 1*C* show that thrombin-stimulated PLC activity was abolished in membranes from PTX-treated cells. This finding and the findings of Fig. 1*B* are consistent with data from platelets (14), which show that the thrombin receptor couples to the PTX-sensitive Gi family of G proteins. To examine the type of α subunit coupling to the ET-1 receptor, we incubated membranes with QL, an antibody directed to the carboxyl terminus of the Gq/G11 α subunit (Fig. 1*D*). The QL antibody has been demonstrated to inhibit responses to Gq-coupled receptors, but not those coupled through Gi (8). When the QL antibody is incubated with membranes prepared from control Rat-1 cells, the ability of ET-1 to activate PLC was inhibited (Fig. 1*D*). As expected (9, 13), the inhibition was completely reversed by addition of the peptide containing the epitope to the assay (Fig. 1*D*). This finding indicates that the Gq/G11 proteins couple ET-1 receptors to PLC in these cells. Very similar results were obtained with Rat-1 v-*src* cells (data not shown). Overall, the data in Fig. 1 indicate that the ET-1 and thrombin receptors in Rat-1 cells generate InsP_3 by coupling to different members of the G protein family; thrombin receptors to the Gi class and ET-1 receptors to the Gq class. This finding provides a potential explanation for the observation that effects of the v-*src* oncogene are only observed when the cells are stimulated with ET-1. Moreover, the data suggest that a potential target of the v-*src* protein may be the receptor–Gq/G11-PLC signaling complex in the membrane.

The α Subunit of Gq/G11 is Phosphorylated on Tyrosine. To determine whether v-*src* transformation induces any change at the level of G protein expression, a quantitative Western blotting protocol was developed using the anti-Gq/G11 antibody QL. Since pp60$^{v\text{-}src}$ is a constitutively active tyrosine kinase (15), the possibility that the α subunit of Gq/G11 might be phosphorylated was also examined. Fig. 2 presents Western blots of the Gq/G11 α subunit immunoprecipitated from extracts of control and v-*src*-transformed Rat-1

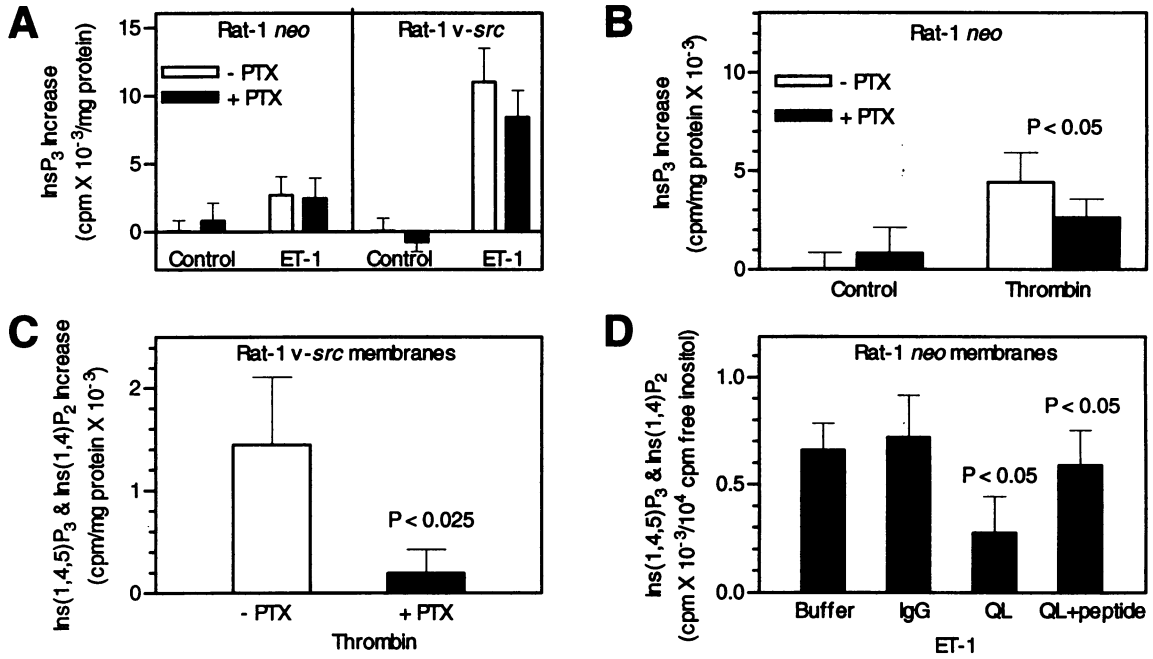

FIG. 1. Effect of PTX on the response of Rat-1 cells to ET-1 and thrombin. (*A*) Rat-1 *neo* and Rat-1 v-*src* cells were treated with 100 ng of PTX per ml (dark bars) and stimulated with 20 nM ET-1 as described. In the presence of LiCl, the Ins(1,3,4)P_3 isomer predominates at 5 min, thus the counts in the resolved Ins(1,4,5)P_3 and Ins(1,3,4)P_3 isomers were combined (shown as InsP_3) and presented as fold over control. The control level of InsP_3 (1964 cpm per mg of protein in Rat-1 *neo* cells and 4360 cpm per mg protein in Rat-1 v-*src* cells) was subtracted from each data set and shown as zero in each panel. (*B*) Treatment of intact Rat-1 *neo* cells with PTX. [^{3}H]*myo*-Inositol-labeled Rat-1 *neo* cells were treated with PTX, stimulated with 1 unit of thrombin per ml for 5 min and the data analyzed as in Fig. 1A. (*C*) Effects of PTX on thrombin-stimulated PLC activity in membranes of Rat-1 v-*src* fibroblasts. [^{3}H]*myo*-Inositol labeled Rat-1 v-*src* cells were pretreated with PTX and membranes prepared before assay for thrombin-stimulated PLC activity. The free [Ca^{2+}] was 100 nM. The levels of Ins(1,4,5)P_3 and inositol 1,4-trisphosphate were combined as an indication of agonist activity because the Ins(1,4,5)P_3 5-phosphatase is very active in membrane preparations. The effect of thrombin in stimulating Ins(1,4,5)P_3 and inositol 1,4-trisphosphate release from the membrane was significantly inhibited by PTX pretreatment ($P < 0.025$). (*D*) Effect of Gq/G11-directed antibody QL on PLC activity in membranes of Rat-1 fibroblasts. Membranes were prepared from Rat-1 cells as described in Fig. 1C except the cells were incubated with 34 μCi of [^{3}H]*myo*-inositol and without PTX treatment. Membranes were incubated with either buffer, a control IgG, QL, or QL plus a C-terminal Gq/G11 peptide containing the epitope for QL and stimulated with 20 nM ET-1 for 5 min (5). The QL antibody significantly reduced the effect of ET-1 ($P < 0.05$) and the peptide significantly reversed the inhibition ($P < 0.05$). Data in *A–D* are mean ± SEM from three to four independent experiments. All statistical differences were determined using the unpaired *t* test.

fibroblasts using the QL antibody. When the immunoprecipitates were blotted with the QL antibody itself, the Gq/G11 α subunit was readily detected, but no difference was observed in the expression of this protein (Fig. 2, *Left*). It is important to stress that the same result was observed regardless of the detergent used to solubilize the cells, whether Gq/G11 was extracted from membranes or intact cells, or whether one- or two-dimensional electrophoresis was used to resolve the proteins (data not shown). No Gq/G11 α subunit was detected when a control IgG protein was used for immunoprecipitation. Thus, transformation of the Rat-1 fibroblast by the v-*src* oncogene does not alter the amount of the Gq/G11 α subunit in the cells. However, when the same immunoprecipitates were blotted with an anti-phosphotyrosine antibody 4G10, the Gq/G11 α subunit (the 42-kDa protein) immunoprecipitated from v-*src*-transformed cells reacted strongly (Fig. 2, *Right*). This result was confirmed using another anti-phosphotyrosine antibody, 6G9. When two-dimensional electrophoresis was used to resolve the proteins, the Gq/G11 α subunit focused as a multiply charged species. In this gel system, the protein from v-*src*-transformed fibroblasts focused at a more acidic isoelectric point than the protein from the control cells, suggesting a high stoichiometry of phosphorylation (data not shown).

To confirm that Gq/G11 is phosphorylated on tyrosine and to determine if there is alteration of serine/threonine phosphorylation after v-*src* transformation, we metabolically labeled the control and transformed cells with [^{32}P]PO_4^{3-} and immunoprecipitated the Gq/G11 α subunit from cell extracts using the QL antibody (Fig. 3*A*). Fig. 3*A* (*Left*) presents autoradiographs of the immunoprecipitates as resolved on a one-dimensional gel. The dotted area indicates the position of the labeled Gq/G11 α subunit and a faster migrating protein that coimmunoprecipitated with the α subunits. To resolve these proteins, the bands in the boxed area were excised from the gel and rerun on a second SDS/polyacrylamide gel. This system completely resolved the proteins (*Right*); the identity of the upper band as the α subunit of Gq/G11 was confirmed by Western blotting with the QL antibody and sequence analysis using mass spectrometry (data not shown). The lower band did not react with the QL antibody. To determine the residue at which phosphorylation occurs, phosphoamino acid analysis was performed. Fig. 3*B* presents an autoradiograph made from the cellulose plates used to resolve the amino acids. The α subunit of Gq/G11 from both control and transformed cells showed minor ^{32}P-incorporation into serine residues, possibly due to background contamination. However, note that the Gq/G11 α subunit from v-*src*-transformed Rat-1 cells showed a significant amount of ^{32}P-incorporation into tyrosine residues. This result provides strong, independent confirmation that v-*src* transformation causes tyrosine phosphorylation of the Gq/G11 α subunit.

Gq/G11 from v-*src*-Transformed Rat-1 Cells Is More Active in a PLC Assay. To determine if the phosphorylation of the Gq/G11 α subunit induces any functional change in its activity and contributes to the ET-1 hyperresponse shown in Fig. 1, we assayed the ability of Gq/G11 to activate PLC-β in a reconstitution experiment (12). Thus, we prepared lipid vesicles using [^{3}H]PtdInsP_2 as substrate, incorporated the Gq/G11 extracted from either control or transformed cells into the vesicles, added purified PLC-β, and measured PLC activity in

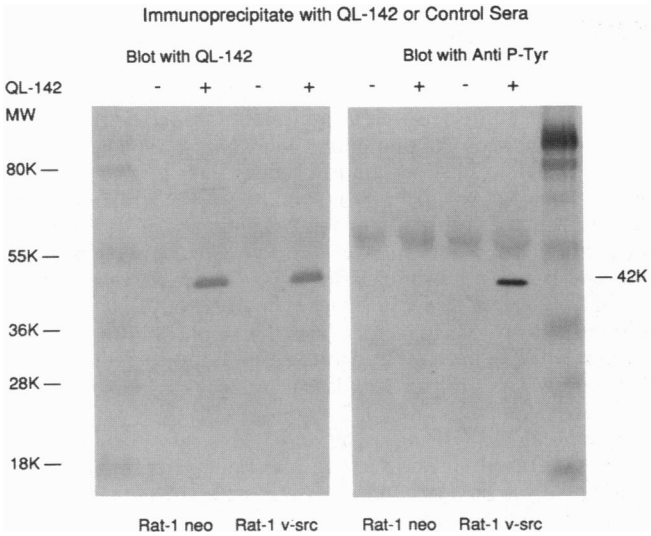

FIG. 2. Western blotting of the Gq/G11 α subunit extracted from control or v-*src*-transformed Rat-1 fibroblasts. (*Left*) The Gq/G11 α subunit in RIPA extracts of the fibroblasts was immunoprecipitated with the Gq/G11 antibody QL (QL-142) and immunoblotted with QL. (*Right*) The Gq/G11 α subunit in the extracts was immunoprecipitated with QL and blotted with an anti-phosphotyrosine (Anti P-Tyr) antibody, 4G10. Cell extracts immunoprecipitated with control IgG antibody (−) or with QL (+); 42K indicates the position of the Gq/G11 α subunit. Results are from a representative experiment performed five times.

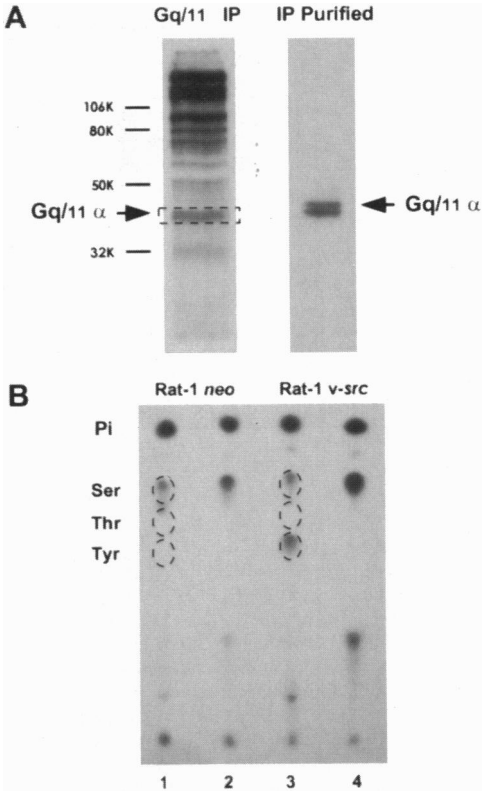

FIG. 3. Phosphoamino acid analysis of the Gq/G11 α subunit. (*A*, *Left*) Autoradiograph of Gq/G11 α subunit immunoprecipitated from $^{32}PO_4^-$-labeled Rat-1 v-*src* cells. The box indicates the position from which the Gq/G11 α subunit and a similarly migrating band were cut from the gel. (*Right*) Radiolabeled Gq/G11 α was resolved on a second SDS/9% acrylamide gel. (*B*) Phosphoamino acid analysis of Gq/G11 α subunit immunoprecipitated from ^{32}P-labeled Rat-1 *neo* and Rat-1 v-*src* cells. Lanes 1 and 3 represent the α subunit of Gq/G11 from control and transformed Rat-1 cells, while lanes 2 and 4 are from the faster migrating protein. Pi, free [^{32}P]phosphate; Ser, serine; Thr, threonine; and Tyr, tyrosine. Results shown are from a representative experiment performed three times.

the presence and absence of the activator, AlF_4^-. Fig. 4 (*Left*) presents the effect of AlF_4^- on Gq activity using a recombinant Gq α subunit as a positive control. It shows that, when recombinant Gq α is activated by AlF_4^-, there is a marked increase of PtdInsP_2 hydrolysis by PLC. Fig. 4 (*Middle* and *Right*) demonstrates the ability of the Gq/G11 α subunit extracted from control and transformed fibroblasts to stimulate exogenous PLC activity. It is important to note that Western blots using the QL antibody showed equal amounts of the Gq/G11 α subunit were extracted from either Rat-1 *neo* or Rat-1 *src* cells (see Fig. 2 and its legend). Extracts of both control and v-*src*-transformed cells had a low basal PLC-stimulating activity in the presence of purified PLC-β alone. However, when AlF_4^- was added, extracts from Rat-1 v-*src* cells showed higher PLC-stimulating activity than those from control cells. The *inset* shows the PLC-stimulating activity from control and v-*src*-transformed cells with the background (open bars) subtracted. Analogous results were obtained if we extracted the Gq/G11 from membranes prepared from control and transformed cells with 1% cholate or if the membranes themselves were washed to remove the endogenous PLC, incubated with exogenous PLC, and stimulated with GTP-γ-S (data not shown). In all cases, the PLC-stimulating activity cells from transformed cells was greater than that from control cells.

To confirm that the Gq/G11 in the cell extracts was responsible for the activation of PLC shown in Fig. 4, we immunodepleted the extracts of Gq and G11 using the QL antibody before adding the mixture to the reconstitution assay. Western blots demonstrated that the amount of QL antibody used in these experiments was able to immunoprecipitate ≈81% of the Gq/G11 α subunit from the cell extracts (data not shown). The data in Fig. 5 show that this treatment greatly decreased the ability of the extract to stimulate PLC activity in the presence of AlF_4^-. The *inset* shows the AlF_4^- stimulated PLC activity with or without immunodepletion by the QL antibody. The PLC-stimulating activity was reduced 88% by depleting most of the Gq/G11 using QL. This result indicates that Gq/G11 is the protein that activates PLC in the reconstitution assay. Taken together, the data in Figs. 1–5 demonstrate that transformation of Rat-1 cells with the v-*src* oncogene results in phosphorylation of the Gq/G11 α subunit on tyrosine residues and that this event correlates with an increase in the ability of the protein to activate PLC.

To further explore the relationship between tyrosine phosphorylation and Gq/G11 α subunit activity, we used herbimycin A, a potent inhibitor of pp60$^{v\text{-}src}$ tyrosine kinases (16), to attempt to block the tyrosine phosphorylation of Gq/G11. A Western blot using the anti-phosphotyrosine antibody demonstrates a dramatic reduction of the phosphorylation of immunoprecipitated pp60$^{v\text{-}src}$ and modest reduction of the Gq/G11 α phosphorylation. In a parallel experiment, herbimycin A also modestly decreased the PLC-stimulating activity of the Gq/11 α subunits extracted from v-*src*-transformed Rat-1 cells (data not shown), suggesting that changes in the tyrosine phosphorylation of the Gq/G11 α subunit are directly linked to their activity in the PLC assay.

DISCUSSION

A number of investigators have found that members of the G protein family can be phosphorylated on both serine and tyrosine residues. The majority of these reports demonstrated the phosphorylation of G protein α subunits *in vitro* using purified or partially purified serine or tyrosine kinases (17–23). Thus, the α subunits of mammalian Gi, Gt, Go, Gs, Gz, and the *Dictyostelium* G protein G2 have been demonstrated to be

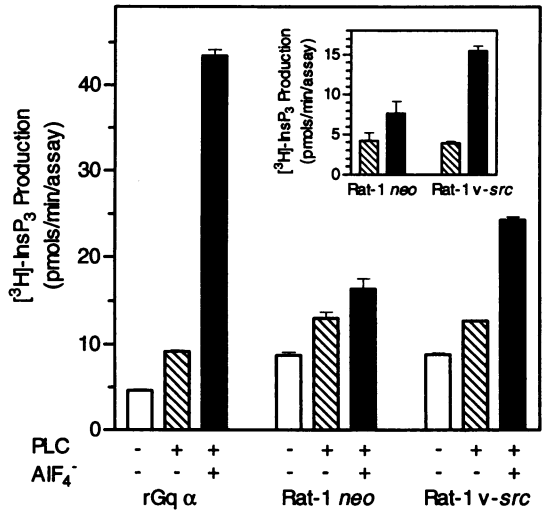

FIG. 4. Comparison of the Gq/G11 activity from RIPA extracts of control or v-*src*-transformed Rat-1 cells in an *in vitro* PLC assay. The open bars show the basal activity of the system without added PLC, the hatched bars show the PLC activity in the absence of stimulation, and the filled bars show the PLC activity in the presence of AlF_4^- (20 μM $AlCl_3$ and 10 mM NaF). The *inset* shows the PLC-stimulating activity in both cell lines with background (no added PLC) subtracted. rGq α stands for a cholate extract of Sf9 cells expressing the α subunit of recombinant Gq using the baculovirus/Sf9 insect cell system. Results shown are mean ± SD from duplicate points in a representative experiment. The experiment was performed three times.

substrates for serine kinases *in vitro* (17–20). The Gi, Gt, Go, and Gs α subunits have also been demonstrated to be *in vitro* substrates for the tyrosine kinases of the insulin receptor or $pp60^{v\text{-}src}$ (21–23). Few of these studies correlated the phosphorylation of the G proteins with functional changes. However, recent studies focusing on the phosphorylation of the Gi α subunit have demonstrated functional consequences. Thus, in cultured NG-108–15 cells and isolated hepatocytes, activation of protein kinase C by phorbol myristic acid prompted an increase in $[^{32}P]PO_4^{3-}$ incorporation into Giα2 and attenuated the ability of agonists to inhibit adenylate cyclase (19, 24). In platelets, the ability of phorbol esters to reduce agonist induced Ca^{2+} mobilization responses also correlated with increased phosphorylation of the Gi α subunit (25). Pretreatment of alveolar macrophages with cholera toxin showed an increase in Giα phosphorylation and a decrease of Gi functional activity. These effects can be blocked by a specific inhibitor of protein kinase A (26). Thus, phosphorylation of the Giα subunit, perhaps by protein kinase C or protein kinase A, appears to be able to attenuate cellular responses, although the mechanism is unknown.

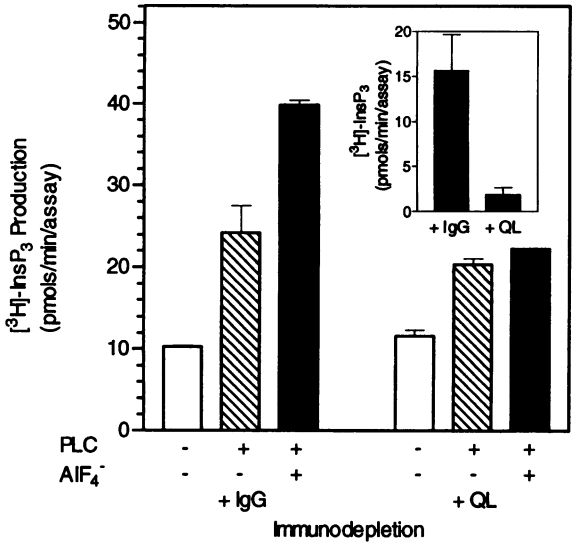

FIG. 5. Activity assay for cell extracts depleted of the Gq/G11 protein. RIPA extracts of transformed Rat-1 cells were immunoprecipitated with the anti-Gq/G11 antibody (QL) or a control antibody (IgG). The ability of the Gq/G11 to activate exogenously added PLC was measured as described in legend to Fig. 4 before and after immunodepletion. (*Left*) Gq/G11 activity in RIPA extracts of Rat-1 v-*src* cells following treatment with IgG. (*Right*) Gq/G11 activity in the same extract after being immunodepleted by the QL antibody. The *inset* shows AlF_4^--stimulated PLC activity (background subtracted) without (+IgG) and with (+QL) immunodepletion by the anti-Gq/G11 antibody QL. Results shown are mean ± SD from duplicate points in a representative experiment. The experiment was performed three times.

Interestingly, G proteins are also substrates for tyrosine kinases. Purified Gi and Go are phosphorylated on tyrosine residues by the purified human insulin receptor (21). The rate of phosphorylation was enhanced when both reactants were incorporated into lipid bilayers, indicating the importance of the environment. In an *in vitro* kinase assay, immunoprecipitated $pp60^{v\text{-}src}$ was able to phosphorylate the purified α subunits of Gs, Gi1, Gi2, Gt, and Go (22). Both of these studies found that the GDP-bound form of the α subunit is the preferred substrate, perhaps because the site of modification is close to the GTP binding site. Our results extend the concept that G proteins can be phosphorylated on tyrosine residues and provide direct evidence for the *in vivo* phosphorylation of the Gq/G11 α subunit on tyrosine residues. Moreover, the finding that the phosphorylated protein extracted from v-*src*-transformed cells is more active in the PLC assay suggests a regulatory role for the tyrosine phosphorylation of the Gq/G11 α subunit. It is important to determine the identity of the kinase that phosphorylates the Gq/G11 α subunit. In this regard, preliminary experiments using an immunoprecipitated $pp60^{v\text{-}src}$ kinase have failed to phosphorylate the Gq/G11 α subunit immunoprecipitated from control Rat-1 cells (data not shown). However, it is known that transformation of Rat-1 cells with the v-*src* oncogene activates multiple protein kinases (27); thus the kinase directly responsible for phosphorylation of the Gq/G11 α subunit may be downstream of a v-*src*-induced signaling cascade.

The effect of phosphorylation on the function of the Gq/G11 the α subunit needs investigation. The α subunit of G proteins has the four following intrinsic activities: the ability to interact with receptors, an ability to bind GTP, a GTP-ase activity that hydrolyzes the bound GTP to GDP, and the ability to stimulate effectors such as PLC when activated (28). It should be emphasized that the tyrosine-phosphorylated Gq/G11 is not constitutively active in the basal, GDP-bound state. Rather, it exhibits an increased ability to stimulate PLC in the GTP-bound state. Apparently, this event can be achieved by receptor-stimulated exchange of GTP for GDP (Fig. 1), AlF_4^- (Fig. 4), or GTP-γ-S (6). Possible biochemical mechanisms for this increased Gq/G11 activity include (*i*) an increase in the GTP-GDP exchange rate of α subunit and (*ii*) better activation of the effector, PLC, by the phosphorylated α subunit of Gq/G11.

Several functional domains of G protein α subunits have been identified, including the receptor-interacting domain, switch regions (I, II, and III), effector domains, $\beta\gamma$-binding regions, and GTP-binding regions. Modification of critical residues of these regions may alter the ability of G proteins to interact with other cellular components, thereby altering the activity of the G protein (see ref. 28 for review). One example is provided by the Gz α subunit. This G protein can be phosphorylated by protein kinase C *in vitro* and *in vivo*, with a major phosphorylation site being Ser-27 (29). This serine is in a domain of the protein thought to be involved in the binding

of the $\beta\gamma$ subunit (28). Indeed, the phosphorylation of the Gz α subunit by protein kinase C inhibits its ability to bind $\beta\gamma$ subunits and, in turn, the phosphorylation is inhibited by $\beta\gamma$ in a dose- and time-dependent fashion (30). Although this study was done *in vitro*, it suggests a mechanism for regulation of Gz activity by preventing reassociation of its subunits. Therefore, mapping of the phosphorylated sites on the Gq/G11 α subunit will be crucial to elucidate mechanism of Gq/G11 regulation.

Although much recent progress has been made in the elucidation of pathways from plasma membrane tyrosine kinases, such as the epidermal growth factor receptor and $pp60^{v\text{-}src}$, through Ras, Raf, and the mitogen-activated protein kinase system to nuclear signaling events (15, 27, 31), a complete picture of how v-*src* induces cell transformation is still elusive. The transformed phenotype may depend not only on the direct effects of the oncogenic product, but also on the ability of the oncogene product to enhance growth factor-dependent signal transduction or to induce the production of growth factors themselves (32, 33). The present results argue that one effect of the $pp60^{v\text{-}src}$ protein is to sensitize Gq-coupled signaling pathways to known mitogens such as ET-1 (34). In addition, there are precedents for the continuous activation of Gq-coupled signaling pathways leading to cell transformation. For example, overexpression of a constitutively active mutant of Gq α (Q209L) in NIH 3T3 cells greatly enhances receptor-stimulated PLC activity (35) and leads to cell transformation (35, 36) and tumor formation in nude mice (36). Expression of this mutant in Rat-1 cells also results in a hyperresponse to serum and an increase in the ability to grow in soft agar (35). Thus chronic amplification of mitogen-stimulated PLC activation could contribute to cell transformation.

Interestingly, there are other important examples of crosstalk between these two major signaling pathways. For example, activation of insulin receptors stimulates the phosphorylation of the β_2-adrenergic receptor on tyrosine residues, an effect that is correlated with a decreased adrenergic response (37, 38). Moreover, Daub *et al.* (39) reported that in Rat-1 cells, stimulation of ET-1, lysophosphatidic acid, or thrombin receptors induces a rapid tyrosine phosphorylation of the epidermal growth factor receptor and the *neu* oncoprotein, suggesting that activation of G protein-coupled receptors can also interact with the receptor tyrosine kinase pathway. Taken together, these data demonstrate that there is considerably more interaction between these two signaling pathways than previously thought.

We thank the following individuals for advice or reagents: Drs. T. K. Harden, J. Boyer (turkey PLC-β), M. Liang (recombinant baculovirus encoding the Gq α), A. Spiegel (QL antibody), P. Sternweis (C-terminal peptide of the Gq α), and J. T. Parsons (anti-phosphotyrosine antibody 6G9). We thank Dr. D. Hunt's laboratory for preliminary mass spectrometry analysis of the Gq α subunit. R.R.M. was a Howard Hughes Medical Institute Predoctoral Fellow during the course of this work. This work was supported by National Institutes of Health Grant PO-1-CA40042.

1. Aaronson, S. A. (1991) *Science* **254,** 1146–1153.
2. Fukami, K., Matsuoka, K., Nakanishi, O., Yamakawa, A., Kawai, S. & Takenawa, T. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9057–9061.
3. Gray, G. M. & Macara, I. G. (1989) *Oncogene* **4,** 1213–1217.
4. Johnson, R. M., Wasilenko, W. J., Mattingly, R. R., Weber, M. J. & Garrison, J. C. (1989) *Science* **246,** 121–124.
5. Mattingly, R. R., Stephens, L. R., Irvine, R. F. & Garrison, J. C. (1991) *J. Biol. Chem.* **266,** 15144–15153.
6. Mattingly, R. R., Wasilenko, W. J., Woodring, P. J. & Garrison, J. C. (1992) *J. Biol. Chem.* **267,** 7470–7477.
7. van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T. & Moolenaar, W. H. (1989) *Cell* **59,** 45–54.
8. Shenker, A., Goldsmith, P., Unson, C. G. & Spiegel, A. M. (1991) *J. Biol. Chem.* **266,** 9309–9313.
9. Gutowski, S., Smrcka, A., Nowak, L., Wu, D. G., Simon, M. & Sternweis, P. C. (1991) *J. Biol. Chem.* **266,** 20519–20524.
10. van der Geer, P. & Hunter, T. (1994) *Electrophoresis* **15,** 544–554.
11. Jelinek, T. & Weber, M. J. (1993) *Biotechniques* **15,** 629–630.
12. Boyer, J. L., Waldo, G. L. & Harden, T. K. (1992) *J. Biol. Chem.* **267,** 25451–25456.
13. Jouneaux, C., Mallat, A., Serradeil-Le Gal, C., Goldsmith, P., Hanoune, J. & Lotersztajn, S. (1994) *J. Biol. Chem.* **269,** 1845–1851.
14. Wilhelm, B. & Siess, W. (1993) *Eur. J. Biochem.* **216,** 81–88.
15. Parsons, J. T. & Weber, M. J. (1989) *Curr. Top. Microbiol. Immunol.* **147,** 79–127.
16. Uehara, Y., Murakami, Y., Sugimoto, Y. & Mizuno, S. (1989) *Cancer Res.* **49,** 780–785.
17. Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jakobs, K. H. (1985) *Eur. J. Biochem.* **151,** 431–437.
18. Lounsbury, K. M., Casey, P. J., Brass, L. F. & Manning, D. R. (1991) *J. Biol. Chem.* **266,** 22051–22056.
19. Houslay, M. D. (1993) in *GTPase in Biology*, eds. Dickey, B. F. & Birnbaumer, L. (Springer, Berlin), Vol. 2, pp. 147–165.
20. Chen, M. Y., Devreotes, P. N. & Gundersen, R. E. (1994) *J. Biol. Chem.* **269,** 20925–20930.
21. Krupinski, J., Rajaram, R., Lakonishok, M., Benovic, J. L. & Cerione, R. A. (1988) *J. Biol. Chem.* **263,** 12333–12341.
22. Hausdorff, W. P., Pitcher, J. A., Luttrell, D. K., Linder, M. E., Kurose, H., Parsons, S. J., Caron, M. G. & Lefkowitz, R. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5720–5724.
23. Moyers, J. S., Linder, M. E., Shannon, J. D. & Parsons, S. J. (1995) *Biochem. J.* **305,** 411–417.
24. Strassheim, D. & Malbon, C. C. (1994) *J. Biol. Chem.* **269,** 14307–14313.
25. Yatomi, Y., Arata, Y., Tada, S., Kume, S. & Ui, M. (1992) *Eur. J. Biochem.* **205,** 1003–1009.
26. Levistre, R., Berguerand, M., Bereziat, G. & Masliah, J. (1995) *Biochem. J.* **306,** 765–769.
27. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. & Soltoff, S. (1991) *Cell* **64,** 281–302.
28. Neer, E. J. (1995) *Cell* **80,** 249–257.
29. Lounsbury, K. M., Schlegel, B., Poncz, M., Brass, L. F. & Manning, D. R. (1993) *J. Biol. Chem.* **268,** 3494–3498.
30. Fields, T. A. & Casey, P. J. (1995) *J. Biol. Chem.* **270,** 23119–23125.
31. Thomas, G. (1992) *Cell* **68,** 3–6.
32. Sporn, M. B. & Roberts, A. B. (1985) *Nature (London)* **313,** 745–747.
33. Wasilenko, W. J., Payne, D. M., Fitzgerald, D. L. & Weber, M. J. (1991) *Mol. Cell. Biol.* **11,** 309–321.
34. MacNulty, E. E., Plevin, R. & Wakelam, M. J. (1990) *Biochem. J.* **272,** 761–766.
35. De Vivo, M., Chen, J., Codina, J. & Iyengar, R. (1992) *J. Biol. Chem.* **267,** 18263–18266.
36. Kalinec, G., Nazarali, A. J., Hermouet, S., Xu, N. & Gutkind, J. S. (1992) *Mol. Cell. Biol.* **12,** 4687–4693.
37. Hadcock, J. R., Port, J. D., Gelman, M. S. & Malbon, C. C. (1992) *J. Biol. Chem.* **267,** 26017–26022.
38. Baltensperger, K., Karoor, V., Paul, H., Ruoho, A., Czech, M. P. & Malbon, C. C. (1996) *J. Biol. Chem.* **271,** 1061–1064.
39. Daub, H., Weiss, F. U., Wallasch, C. & Ullrich, A. (1996) *Nature (London)* **379,** 557–560.