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Biochemistry

Tyrosine phosphorylation of G protein α subunits by pp60$^{c\text{-}src}$

{guanosine 5′-[γ-thio]triphosphate binding/GTPase activity/stimulatory guanine nucleotide-binding regulatory protein α subunit (H21a)}

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ABSTRACT **A number of lines of evidence suggest that cross-talk exists between the cellular signal transduction pathways involving tyrosine phosphorylation catalyzed by members of the pp60$^{c\text{-}src}$ kinase family and those mediated by guanine nucleotide regulatory proteins (G proteins). In this study, we explore the possibility that direct interactions between pp60$^{c\text{-}src}$ and G proteins may occur with functional consequences. Preparations of pp60$^{c\text{-}src}$ isolated by immunoprecipitation phosphorylate on tyrosine residues the purified G-protein α subunits (Gα) of several heterotrimeric G proteins. Phosphorylation is highly dependent on G-protein conformation, and Gα(GDP) uncomplexed by βγ subunits appears to be the preferred substrate. In functional studies, phosphorylation of stimulatory Gα (Gα$_s$) modestly increases the rate of binding of guanosine 5′-[γ-[^{35}S]thio]triphosphate to G$_s$ as well as the receptor-stimulated steady-state rate of GTP hydrolysis by G$_s$. Heterotrimeric G proteins may represent a previously unappreciated class of potential substrates for pp60$^{c\text{-}src}$.**

The oncogenes of many retroviruses encode tyrosine-specific protein kinases that are presumed to phosphorylate cellular proteins involved in the control of cell growth and differentiation. Many of these kinases belong to the src family of kinases by virtue of their structural homology with the oncogene product (pp60$^{v\text{-}src}$) encoded by Rous sarcoma virus. Members of this family are cytoplasmic proteins that are myristoylated, associated with cellular membranes, and may mediate the actions of diverse cell-surface receptors (1–3).

A number of lines of experimental evidence point to the existence of functional interactions between activated members of the pp60$^{c\text{-}src}$ family and elements in the signal transduction pathways mediated by guanine nucleotide-binding regulatory proteins (G proteins). For example, several growth factors and lymphocyte cell-surface antigens whose actions have been proposed to be mediated by members of the src subfamily (1–9) also modulate G-protein functioning (10, 11) and adenylyl cyclase activity (12, 13). In addition, two enzymes involved in inositol phospholipid metabolism, which is highly regulated by G proteins, may be targets of phosphorylation by activated *src* gene products (1, 14). Furthermore, overexpression of avian pp60$^{c\text{-}src}$ in fibroblasts results in enhanced intracellular cAMP levels and adenylyl cyclase activity in response to β-adrenergic hormones, which act via the stimulatory G protein (G$_s$) (15, 16).

The molecular nature of these putative interactions has remained obscure. However, the observation that pp60$^{c\text{-}src}$ is a major contaminant in some highly purified G-protein preparations (17) suggests the possibility of a direct association between pp60$^{c\text{-}src}$ and G proteins. In this study, we examined the abilities of purified G proteins to serve as substrates for phosphorylation by pp60$^{c\text{-}src}$.

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EXPERIMENTAL PROCEDURES

Purification and Reconstitution of β$_2$-Adrenergic Receptors (β$_2$AR) and G Proteins. Purification of β$_2$AR from hamster lung (18), rhodopsin, and transducin from bovine retina (19), βγ subunits from bovine brain (20, 21), and G-protein α subunits (Gα) expressed in *Escherichia coli* (22–24) were as described. Receptors were reconstituted in phosphatidylcholine vesicles by using octyl glucoside (25).

pp60$^{c\text{-}src}$ Immunoprecipitations and *in Vitro* Kinase Assays. C3HT10½ (C5) or Syrian hamster (4A) cells overexpressing wild-type avian pp60$^{c\text{-}src}$ at levels >25-fold over nontransfected cells (15) were pretreated with 1.6 μM phorbol 12-myristate 13-acetate for 15 min, the cells were lysed (1.5 ml per 75-cm^2 flask) in RIPA [150 mM NaCl/50 mM Tris·HCl, pH 7.5/1% Nonidet P-40/0.25% deoxycholate/peptide inhibitors (18)/1 μM microcystin (Calbiochem)] plus 0.1% SDS, and the insoluble fraction was removed by 10 min of centrifugation at 12,000 × *g*. Phorbol pretreatment (26), although not required, enhanced the phosphorylating activity of pp60$^{c\text{-}src}$ (data not shown).

Immunoprecipitations were performed by using the rodent/avian pp60$^{c\text{-}src}$-specific monoclonal antibody GD11 (27). Specifically, 20 μl of a 1:10 dilution of GD11 in Dulbecco's phosphate-buffered saline (PBS) was incubated per 1.5 ml of solubilized extract on ice for 1 hr. At this point, 25 μl of Pansorbin (Calbiochem) prewashed in bovine serum albumin (1.5 mg/ml) was added, and the mixture was incubated for 30 min on ice and centrifuged at 12,000 × *g* for 3 min at 4°C. Immunoprecipitates were washed sequentially with RIPA plus 0.1% SDS, with RIPA without SDS, with 500 mM LiCl/0.1% Nonidet P-40/50 mM Tris·HCl, pH 7.5 (twice), and with PBS. They were then resuspended in 100 μl of 20 mM Hepes, pH 7.0/1–5 mM $MnCl_2$/5 mM GDP/10% (vol/vol) glycerol/3–8 pmol of purified Gα. Reactions were started by addition of 600 μM $MgCl_2$/60 μM [γ-^{32}P]ATP (1000–2000 cpm/pmol) and were conducted at 30°C. For determinations of phosphorylation stoichiometry, reactions were terminated by addition of SDS/PAGE sample buffer, and stoichiometry was assessed by excision of gel bands after PAGE and was quantitated by Cerenkov counting. Before incubation in the kinase assay mixture, 30–70% of the Gα

Abbreviations: G protein, guanine nucleotide-binding regulatory protein; G$_s$, stimulatory G protein; G$_i$, inhibitory G protein; G$_t$, retinal G-protein transducin; β$_2$AR, β$_2$-adrenergic receptor(s); Iso, (−)-isoproterenol; Alp, (±)-alprenolol; Gα, G-protein α subunit(s); Gα$_{ss}$, α subunit (short form) of G$_s$; Gα$_{sL}$, α subunit (long form) of G$_s$; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

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protein (by mass) was active—i.e., bound guanosine 5′-[γ-thio]triphosphate (GTP[γS]). Since inactive Gα is not a substrate for phosphorylation (see Fig. 2), all stoichiometries were calculated relative to the active protein initially present in the kinase mixture.

For functional studies, nonradiolabeled phosphorylated $G\alpha_s$ was prepared and reactions were terminated by addition of a 2× vol of ice-cold 10 mM Tris, pH 8/5 mM EDTA. To produce unphosphorylated $G\alpha_s$, $G\alpha_s$ was incubated in one of three mock kinase mixtures; either AppNHp was substituted for ATP, or the GD11 antibody had not been preincubated with cell lysate, or the GD11 immune complex was replaced by the immune complex of the unrelated antibody SP20 (unable to precipitate $pp60^{c\text{-}src}$; ref. 27). Pansorbin was removed by centrifugation at 12,000 × *g* for 3 min; under these conditions ≈70% of $G\alpha_s$ remained in the supernatant. Equivalent amounts of phosphorylated or unphosphorylated $G\alpha_s$ (determined by the ultimate extent of GTP[γ-^{35}S] binding) were subsequently examined in functional assays.

GTPase Assay. For assays of β_2AR-catalyzed activity, vesicles containing 200 fmol of reconstituted β_2AR were incubated with 200 fmol of recombinant $G\alpha_s$ and 1 pmol of βγ subunits. For assays of rhodopsin-catalyzed activity, 200 fmol of bovine rhodopsin was preincubated with 0.25–1 pmol of retinal G-protein transducin (G_t) in the same buffer in the dark. GTPase assays were conducted at 30°C as described (18, 28). Stimulation by the β-adrenergic agonist (−)-isoproterenol (Iso) was generally 2- to 3-fold, and stimulation by light was 10- to 12-fold over basal in their respective systems. To ensure the absence of active rhodopsin, the basal GTPase activity of G_t was determined in the light with denatured rhodopsin (microwave on high for 3 min).

GTP[γS] Binding. Fifty microliters of vesicles containing 600 fmol of reconstituted β_2AR was incubated with 300 fmol of the short form of bovine $G\alpha_s$ ($G\alpha_{ss}$) and 300 or 1500 fmol of βγ subunits in 20 mM Hepes, pH 8.0/100 mM NaCl/0.1 mM ascorbic acid/1–10 mM $MgCl_2$/0.1 μM GTP[γ-^{35}S] (0.2–0.5 μCi per tube; 1 Ci = 37 GBq) in the presence of 30 μM Iso or 5 μM (±)-alprenolol (Alp; a β-adrenergic antagonist) (total vol, 100 μl) and incubated at 30°C. To assess total binding, the buffer was supplemented with 1.0 μM GTP[γS] and 0.1% Lubrol and was incubated for 60 min. Reactions were stopped by addition of ice-cold 20 mM Tris, pH 8.0/1 mM GTP/10 mM $MgCl_2$/100 mM NaCl/0.1% 2-mercaptoethanol/0.1% Lubrol. Bound GTP[γ-^{35}S] was determined by gel filtration on G-50 columns.

Phosphoamino Acid Analyses. ^{32}P-labeled proteins eluted from SDS/polyacrylamide gels were hydrolyzed by acid (29) and phosphoamino acids were separated by electrophoresis and ascending chromatography (30).

RESULTS

Phosphorylation of G Proteins by $pp60^{c\text{-}src}$. We used $pp60^{c\text{-}src}$ that had been immunoprecipitated from $pp60^{c\text{-}src}$ overexpressing cells (see *Experimental Procedures*) as a source of kinase activity. The recombinant $G\alpha_{ss}$ (22) is rapidly phosphorylated in this *in vitro* kinase assay, as were recombinant $G\alpha_{sL}$ (L, long form), $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ (i, inhibitory), and $G\alpha_o$, as well as purified bovine transducin (Fig. 1 *A* and *B*). Stoichiometries of 0.3–0.9 mol of PO_4 per mol of Gα were routinely achieved. In contrast, β_2AR did not appear to be a substrate for the kinase (data not shown).

This G-protein phosphorylation occurs exclusively on tyrosine residues. Thus, the ^{32}P incorporated into Gα is not removed by treatment with strong alkali (Fig. 1 *A* and *B*), and phosphoamino acid analyses of $G\alpha_s$ and $G\alpha_{i2}$ reveal only phosphotyrosine (Fig. 1 *C* and *D*).

Factors Affecting Phosphorylation. The phosphorylation stoichiometries obtained with G_s and G_{i2} purified from bovine erythrocytes were consistently lower than those found with the recombinant versions (data not shown). We therefore tested the influence of βγ on phosphorylation. Fig. 2*A* shows that preincubation of recombinant $G\alpha_{ss}$ with increasing concentrations of βγ subunits inhibits subsequent phosphorylation of the α subunit in a dose-dependent manner. This effect is not due to differences in detergent content on βγ-subunit addition, since all assays were conducted under identical buffer and detergent conditions. The lack of effect of βγ subunit on autophosphorylation of $pp60^{c\text{-}src}$ suggests that the inhibition observed is due to formation of the G_s heterotrimer.

The activation status of $G\alpha_{ss}$ also affected its ability to serve as a substrate. Thus, heat-inactivated $G\alpha_{ss}$, which has largely lost its ability to bind guanine nucleotides, is poorly phosphorylated (Fig. 2*B*). Furthermore, pretreatment of $G\alpha_{ss}$

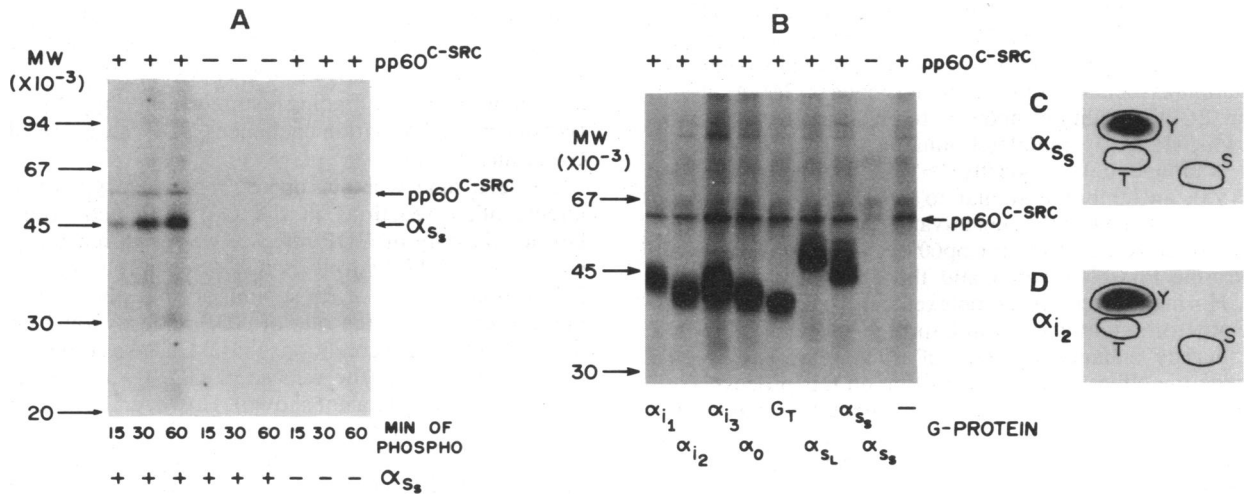

FIG. 1. Tyrosine phosphorylation of Gα proteins by $pp60^{c\text{-}src}$. Avian $pp60^{c\text{-}src}$ and [γ-^{32}P]ATP were incubated at 30°C with 5 pmol of either recombinant human $G\alpha_{ss}$ for 15–60 min (*A*) or with purified bovine retinal G_t, recombinant human $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_{ss}$, and $G\alpha_{sL}$ for 45 min (*B*); samples were analyzed by SDS/10% PAGE. As controls, $G\alpha_{ss}$ was incubated with the GD11 antibody alone (lanes $-pp60^{c\text{-}src}$), and $pp60^{c\text{-}src}$ was incubated without $G\alpha_{ss}$ (lanes $-\alpha_{ss}$). Autoradiograms from gels that had been treated with 1 M KOH are shown. Some phosphorylation could also be obtained with endogenous mammalian $pp60^{c\text{-}src}$ (data not shown). $G\alpha_{ss}$ (*C*) and $G\alpha_{i2}$ (*D*) were excised from similar gels that had not been treated with KOH, and phosphoamino acid composition was determined. Circled are phosphothreonine (T), phosphoserine (S), and phosphotyrosine (Y) standards.

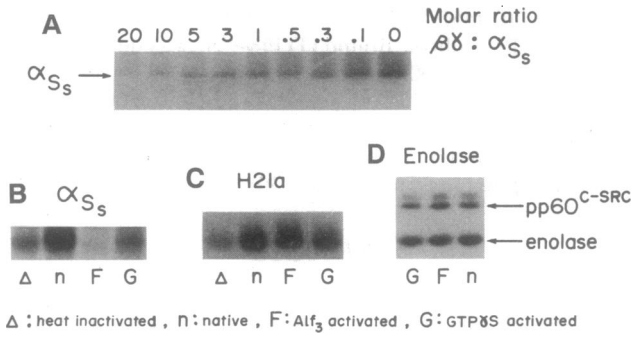

FIG. 2. Conformational dependence of $G\alpha_{ss}$ phosphorylation. (*A*) Purified recombinant $G\alpha_{ss}$ (5 pmol) was preincubated for 15 min at 4°C with various concentrations of $\beta\gamma$ subunit (presented as molar ratios relative to $G\alpha_{ss}$) and incubated with $pp60^{c\text{-}src}$ at 30°C in the kinase assay mixture. Purified recombinant $G\alpha_{ss}$ (*B*), purified recombinant $G\alpha_s$ (H21a) mutant (*C*), or 10 μg of enolase (*D*) was incubated in the kinase assay mixture with $pp60^{c\text{-}src}$ (lanes n) and [γ-^{32}P]ATP for 45 min at 30°C. Alternatively, substrates were partially inactivated by preincubation for 30 min at 90°C (lanes Δ), or with 10 mM NaF (lanes F) or 1 μM GTP[γS] and 5 mM $MgCl_2$ (lanes G) for 45 min at 4°C before incubation with the kinase. Representative autoradiograms are shown.

or $G\alpha_{i2}$ (data not shown) with 1 μM GTP[γS] or 10 mM NaF diminishes the subsequent phosphorylation attainable in the kinase assay by 70–80% (Fig. 2*B*). NaF activates G proteins since the trace amounts of AlF_4^- present (31) associate with bound GDP to mimic the γ phosphate of GTP (32, 33). In contrast, the ability of $pp60^{c\text{-}src}$ to phosphorylate enolase or to undergo autophosphorylation was unaffected by NaF and only slightly (≈20%) diminished by 1 μM GTP[γS] (Fig. 2*D*); the latter is probably due to competition for the ATP-binding site on the kinase.

Mechanistic Basis of Conformation Dependence. Why do F and GTP[γS] inhibit phosphorylation of $G\alpha_s$? One possibility is that binding of guanine nucleotide triphosphate analogues sterically prevents phosphorylation. Alternatively, the alteration in $G\alpha_s$ conformation that occurs upon guanine nucleotide binding (i.e., activation) renders a tyrosine(s) inaccessible to the kinase.

To distinguish between these alternatives, we examined the phosphorylation characteristics of a recombinant mutant form of $G\alpha_s$ (H21a) (24). A point mutation in $G\alpha_s$ (H21a), while permitting binding of GTP[γS] (and association with AlF_4^-), impairs the ability of the G protein to undergo activation-associated conformational changes (34). As shown in Fig. 2*C*, the ability of $pp60^{c\text{-}src}$ to phosphorylate recombinant $G\alpha_s$ (H21a) is completely unaffected by preincubation with NaF and only modestly (≈30%) inhibited by 1 μM GTP[γS], an inhibition similar to that found with enolase. These results suggest that unactivated $G\alpha_s$ free of $\beta\gamma$ subunit is the preferred substrate for $pp60^{c\text{-}src}$.

Tyrosine Phosphorylation and the Functional Activity of $G\alpha_s$. Hormone–receptor complexes catalyze the release of GDP, the rate-limiting step in G-protein activation (35–37), and thereby enhance the rate of GTP[γ-^{35}S] binding. To examine the effect of phosphorylation on this process, $G\alpha_{ss}$ was first phosphorylated to near stoichiometric levels (≈0.7 mol of P_i per mol of $G\alpha_{ss}$), then combined with equimolar levels of $\beta\gamma$ subunit, and added to phospholipid vesicles containing β_2AR.

As shown in Fig. 3, both basal (+Alp) and stimulated (+Iso) initial rates of GTP[γ-^{35}S] binding were enhanced by phosphorylation of $G\alpha_{ss}$. Under basal conditions (+Alp) 6.0 ± 1.3% of the unphosphorylated $G\alpha_{ss}$ and 13.8 ± 1.4% of the phosphorylated $G\alpha_{ss}$ present in the assay mixture had bound GTP[γS] after 1 min of incubation. In the presence of activated receptor (+Iso), 17.0 ± 1.9% of the unphosphorylated $G\alpha_{ss}$ and 28.4 ± 6.5% of the phosphorylated $G\alpha_{ss}$ had bound GTP[γS] over the same interval (means ± SEM; n = 3).

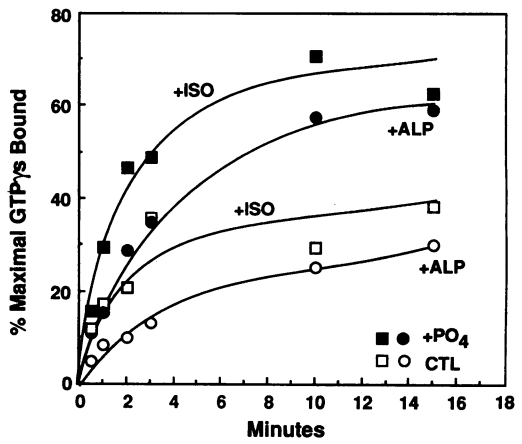

FIG. 3. Effects of tyrosine phosphorylation on $G\alpha_{ss}$ function. Phosphorylated (+PO_4; solid symbols) or unphosphorylated (CTL; open symbols) $G\alpha_{ss}$ was incubated with equimolar levels of $\beta\gamma$ subunit and added to phospholipid vesicles containing β_2AR at 4°C. Binding of GTP[γ-^{35}S] was conducted at 30°C for the indicated times in the presence of agonist (+Iso; squares) or antagonist (+Alp; circles).

As the ultimate extent of GTP[γS] binding remained unaltered by phosphorylation (data not shown), these functional effects of phosphorylation cannot easily be attributed to a hypothetical protection of $G\alpha_s$ against inactivation or degradation in the course of the assays.

Heterotrimeric G_s is normally in equilibrium with its subunits α_s and $\beta\gamma$. The free $G\alpha$ release GDP (and thus bind GTP[γS]) at a significantly faster rate than the holoprotein (35). Therefore, if phosphorylation of $G\alpha_s$ decreases its affinity for $\beta\gamma$ subunit, and thereby increases the relative concentration of free $G\alpha_s$ (versus its heterotrimeric form), it would also increase the overall rate of GTP[γS] binding of the mixture when compared with unphosphorylated preparations. We therefore repeated the GTP[γS] binding assay but in the presence of 5-fold molar excess of $\beta\gamma$ subunit to shift the G-protein equilibrium back toward holoprotein formation and overcome any phosphorylation-induced changes in affinity. Under these conditions, we detected no effect of $G\alpha_s$ phosphorylation on the rate of GTP[γS] binding (n = 2; data not shown). These findings support the hypothesis that tyrosine phosphorylation enchances GDP release by altering $G\alpha\beta\gamma$ interactons.

To determine whether $pp60^{c\text{-}src}$ phosphorylation of $G\alpha_s$ affects other reactions in the G-protein activation cycle besides the rate of GDP release, we measured the steady-state rate of GTP hydrolysis (GTPase) in the presence of a 5-fold molar excess of $\beta\gamma$ subunit so that phosphorylation-induced changes in the rate of GDP release would not be a factor. Under these conditions, basal GTPase activity (whose rate-limiting step is the release of GDP) was unaltered by phosphorylation (data not shown). However, the rate of GTP hydrolysis catalyzed by activated β_2AR was 205 ± 37% of the corresponding value for unphosphorylated $G\alpha_{ss}$ (mean ± SEM; n = 9). Furthermore, the extent of increase in steady-state GTPase activity correlates with the stoichiometry of phosphorylation of $G\alpha_{ss}$ (r = 0.78 ± 0.2; n = 3; data not shown).

Similarly, phosphorylation of transducin enhanced the rate of GTP hydrolysis catalyzed by light-activated rhodopsin to 140 ± 10% of that seen with unphosphorylated G_t (mean ± SEM; n = 3). These results indicate that phosphorylation by

pp60$^{c\text{-}src}$ modulates multiple steps in the G-protein activation cycle.

DISCUSSION

This report documents the ability of pp60$^{c\text{-}src}$ to stoichiometrically phosphorylate the purified Gα subunits of several heterotrimeric G proteins on tyrosine residues. While we have not yet determined the exact sites of phosphorylation, the seven tyrosine residues are highly conserved for each of the Gα subunits tested here (35) as well as in Gα_q (38–40). The negative effects of $\beta\gamma$-subunit association on phosphorylation (and perhaps vice versa) might suggest that phosphorylation by pp60$^{c\text{-}src}$ occurs on tyrosine(s) near the amino terminus, the only region clearly implicated in $\beta\gamma$-subunit interaction (35). However, none of the seven highly conserved candidate residues is located in this region.

Although no consensus amino acid sequence for phosphorylation by pp60$^{c\text{-}src}$ has been established, acidic residues are often located amino terminal to the tyrosine phosphate acceptor (41). Of the seven conserved tyrosines, Tyr-170 and Tyr-312 (of Gα_{sL}) and their conserved counterparts have vicinal acidic residues.

Phosphorylation of G Proteins by Receptor Kinases. G proteins have previously been shown to be substrates for *in vitro* phosphorylation by receptor tyrosine kinases. For example, the purified insulin receptor was shown to tyrosine phosphorylate the α subunits of G_t and other G proteins to a maximum stoichiometry of 0.2 mol of PO_4 per mol of active G protein (42, 43) and to prefer Gα_t to its holotransducin form (42). However, no functional consequences of these phosphorylation events were reported. More recently, Cerione and colleagues (44) demonstrated that a specific low molecular weight GTP-binding protein is a substrate for stoichiometric phosphorylation on tyrosine residues by the epidermal growth factor receptor. This phosphorylation slightly enhances the binding of GTP[γS] but also paradoxically decreases the rate of dissociation of GDP.

In all cases, the binding of GTP[γS] renders the GTP-binding proteins poorer substrates for their respective tyrosine kinases (42–44). Interestingly, the calcium- and phospholipid-dependent protein kinase also preferentially phosphorylates the unliganded and uncomplexed form of Gα_i (45, 46).

In this study, we demonstrate that phosphorylation by pp60$^{c\text{-}src}$ in itself modestly enhances the activities of G_s and transducin. We believe it is likely that we are underestimating the functional effects of *in vitro* phosphorylation on the purified G proteins. Although Gα_s was phosphorylated to a high stoichiometry (0.4–0.9 mol of PO_4 per mol of Gα_s), no attempt was made to remove any residual unphosphorylated Gα_s; thus, functional assessments were performed on somewhat mixed populations of Gα_s. In addition, the functional influence of variables such as the $\beta\gamma$-subunit and Mg^{2+} concentrations has yet to be systematically determined.

Oncogenic G Proteins and v-src. Recent evidence suggests that continuous activation of Gα_s and Gα_{i2} via point mutations can be oncogenic (47, 48). We have observed that pp60$^{v\text{-}src}$, which is constitutively active, also can phosphorylate the α subunits of G proteins *in vitro* (data not shown). Assuming that these phosphorylation events enhance G-protein functioning, these studies may suggest one mechanism of oncogenicity of this viral oncogene. Tantalizing in this context is a report of altered isoelectric points for cholera and pertussis toxin substrates purified from cells that had been transformed by v-src, perhaps consistent with the occurrence of phosphorylation events (49).

Physiological Relevance. This study was not designed to determine whether tyrosine phosphorylation of Gα occurs in intact cells. However, in preliminary experiments we have been able to use anti-phosphotyrosine antibodies to immunoprecipitate proteins from cells that specifically immunoblot with anti-Gα_s antibodies. Moreover, a 75–100% increase in the abundance of such proteins is observed in cells overexpressing pp60$^{c\text{-}src}$. The high stoichiometry of Gα phosphorylation by pp60$^{c\text{-}src}$ observed *in vitro*, its strict substrate conformational requirements, the associated modest enhancements of activity, and the preliminary evidence that tyrosine phosphorylation of Gα_s is associated with pp60$^{c\text{-}src}$ overexpression in intact cells may all imply physiological relevance.

It has been proposed that phosphotyrosines primarily serve to complex proteins with others that contain SH2 (SRC homology) domains (4, 50). Among this latter class of proteins are members of the pp60$^{c\text{-}src}$ family, the GTPase-activating protein (GAP), phospholipase $C_\gamma 1$, phosphatidylinositol 3-kinase (1), and the cytoskeletal protein tensin (51). In this context, phosphorylation of G proteins by pp60$^{c\text{-}src}$, if it occurs in intact cells, could serve to alter their functionality by facilitating interactions with specific effectors, regulatory proteins, or the cytoskeleton. Clearly, much more work is required to directly address this potential cross-talk between two major signal transduction pathways in mammalian cells.

W.P.H. and J.A.P. contributed equally to this work. We thank W. Carl Stone for preparations of β_2AR, Richard Randall and Millie McAdams for peptide synthesis, Juan Codina for purified G proteins, and Ethan Lee for recombinant Gα_s (H21a). M.E.L. acknowledges support from Alfred G. Gilman, National Institutes of Health Grant GM34497, and American Cancer Society Grant BE30M. This work was supported by National Institutes of Health Grant HL16037 (to R.J.L.).

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