Identification of the *in vitro* phosphorylation sites on $G_{s\alpha}$ mediated by pp60^{c-src}

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Overexpression of pp60$^{c\text{-}src}$ in mouse fibroblasts potentiates both agonist-induced signalling through β-adrenergic receptors and cyclic AMP accumulation in response to cholera toxin [Bushman, Wilson, Luttrell, Moyers and Parsons (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 7462–7466; Moyers, Bouton and Parsons (1993) Mol. Cell. Biol. **13**, 2391–2400]. In reconstitution experiments *in vitro*, phosphorylation of $G_{s\alpha}$ by immune-complexed pp60$^{c\text{-}src}$ resulted in enhanced rates of receptor-mediated guanosine 5′-[γ-thio]triphosphate (GTP[S]) binding and GTP hydrolysis [Hausdorff, Pitcher, Luttrell, Linder, Kurose, Parsons, Caron and Lefkowitz (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 5720–5724]. These results suggest that one mechanism by which pp60$^{c\text{-}src}$ affects signalling through the β-adrenergic receptor is by phosphorylation and functional alteration of the G protein. To elucidate how phosphorylation of $G_{s\alpha}$ might affect its function, we subjected phosphorylated, recombinant $G_{s\alpha}$ to tryptic phosphopeptide analysis. Phosphotryptic peptides were purified by h.p.l.c. and analysed by Edman degradation to determine the cycle numbers at which radiolabelled phosphotyrosine was released. Candidate peptides that contained Tyr residues at the corresponding positions were synthesized, phosphorylated *in vitro* by pp60$^{c\text{-}src}$, and their migrations in two-dimensional electrophoresis/t.l.c. were compared with those of tryptic phosphopeptides from intact $G_{s\alpha}$. We report here that $G_{s\alpha}$ is phosphorylated on two residues by pp60$^{c\text{-}src}$, namely, Tyr-37 and Tyr-377. Tyr-37 lies near the site of βγ binding in the N-terminus, within a region postulated to modulate GDP dissociation and activation by GTP [Johnson, Dhanasekaran, Gupta, Lowndes, Vaillancourt and Ruoho (1991) J. Cell Biochem. **47**, 136–146], while Tyr-377 is located in the extreme C-terminus, within a region of $G_{s\alpha}$ important for receptor interaction [Sullivan, Miller, Masters, Beiderman, Heideman and Bourne (1987) Nature (London) **334**, 712–715]. The location of these residues suggests that phosphorylation may affect the function of both of these regulatory domains.

INTRODUCTION

Evidence from several avenues of research indicates that multiple signalling events are influenced by cross-talk between pathways involving tyrosine kinases and heterotrimeric G proteins. For example, heterotrimeric G proteins have been reported to be involved in signalling through tyrosine kinase receptors such as the epidermal growth factor (EGF) and insulin receptors. In rat hepatocytes, EGF has been shown to stimulate inositol phosphate production in a pertussis toxin-sensitive manner, and EGF treatment of isolated hepatocyte membranes results in a stimulation of the initial rates of both [^{32}P]GDP dissociation and [^{35}S]guanosine 5′-[γ-thio]triphosphate (GTP[S]) association in these preparations (Liang and Garrison, 1991). Similarly, EGF was found to stimulate a pertussis toxin-sensitive phospholipase A2 in rat kidney tubule cells (Teitelbaum, 1990) and an increase in cyclic AMP accumulation in perfused rat hearts that was mediated by a stimulatory G protein (Nair et al., 1989). Other studies suggest that tyrosine phosphorylation of G_{β} subunits and translocation of $G_{i\alpha}$ to the nucleus may be involved in platelet-derived growth factor-, EGF-, and insulin-induced mitogenesis (Crouch, 1991).

Substantial evidence also suggests a role for G_{α} proteins in the mechanisms of insulin receptor signalling. Pretreatment of intact adipocytes, BC_3H-1 myocytes and hepatocytes with pertussis toxin alters the metabolic effects of insulin and the insulin-stimulated GTP hydrolysis and GTP[S] binding to a 40 kDa protein of the G_{α} family in myocyte membranes (Luttrell et al., 1990). However, no evidence for tyrosine phosphorylation of G_{α} was found in this study. Similarly, Rothenberg and Kahn (1988) reported that in rat liver membranes, insulin inhibits pertussis toxin-catalysed ADP-ribosylation of G_i, though G_i does not serve as a substrate for phosphorylation by the insulin receptor kinase. These results are suggestive of an indirect interaction between the insulin receptor and G proteins.

In contrast, several studies provide evidence for a direct interaction between G_{α} and the insulin receptor, and indicate that the receptor can phosphorylate G_{α} proteins on tyrosine residues. In reconstituted phospholipid vesicles, the insulin receptor was found to phosphorylate $G_{i\alpha}$ and $G_{o\alpha}$ to stoichiometric levels, with the inactive (GDP-bound) form of the proteins being the preferred substrates (Krupinsky et al., 1988). In addition, Zick et al. (1986) reported that the GDP-bound α subunit of transducin is phosphorylated by purified insulin receptor. Taken together, these findings provide evidence for an involvement of G proteins in mediating some aspects of insulin receptor signalling, although a clear understanding of the mechanism of these interactions is not forthcoming.

A number of studies have also implicated a role for interactions between the non-receptor tyrosine kinases and G proteins. The signalling pathway leading to expression of the transformation-related 9E3 gene by the v-*fps* oncogene appears to include a

Abbreviations used: BCA, bicinchoninic acid; DOC, deoxycholate; EGF, epidermal growth factor; $G_{s\alpha s}$, short form of recombinant bovine $G_{s\alpha}$; GTP[S], guanosine 5′-[γ-thio]triphosphate; $HisG_{s\alpha s}$, hexahistidine-tagged form of $G_{s\alpha s}$; mAb, monoclonal antibody; NP-40, Nonidet P-40; TFA, trifluoroacetic acid; TPCK, tosylphenylalanylchloromethane.

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GTP-binding protein (Alexandropoulos et al., 1991), while activation of phospholipase D in phagocytic leucocytes is synergistically regulated by G proteins and tyrosine kinases (Dubyak et al., 1993). Signalling through the G protein-coupled endothelin-1 receptor is potentiated by pp60$^{v\text{-}src}$ (Mattingly et al., 1992), and treatment of normal mesangial cells with endothelin-1 stimulates autophosphorylation of pp60$^{c\text{-}src}$ (Simonson and Herman, 1993). Akiho et al. (1993) reported the decreased coupling of G_s with adenylate cyclase in v-*src*-transformed NIH-3T3 fibroblasts and provided indirect evidence that $G_{s\alpha}$ is phosphorylated *in vitro* on tyrosine by pp60$^{v\text{-}src}$. In platelets, stimulation with adrenaline (epinephrine) has been shown to induce the association of $G_{i\alpha}$ with pp60$^{c\text{-}src}$ (Torti et al., 1992).

In addition, we have reported that pp60$^{c\text{-}src}$ affects signalling through the β-adrenergic receptor (Bushman et al., 1990; Moyers et al., 1993). Overexpression of pp60$^{c\text{-}src}$ in C3H10T1/2 mouse fibroblasts potentiates the intracellular accumulation of cyclic AMP upon stimulation of the β-adrenergic receptor. Additionally, stimulation of c-*src* overexpressors with cholera toxin results in an enhanced cyclic AMP accumulation (over and above that seen in normal control cells), suggesting that the $G_{s\alpha}$ protein may be altered in c-*src* overexpressors.

Related studies have shown that immune-complexed pp60$^{c\text{-}src}$ is capable of phosphorylating purified recombinant $G_{s\alpha}$, as well as other G_α subtypes ($G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, $G_{o\alpha}$, and $G_{\alpha T}$), to near stoichiometric levels on tyrosine residues (Hausdorff et al., 1992). Phosphorylation of $G_{s\alpha}$ is inhibited in the presence of increasing concentrations of $\beta\gamma$ and preferentially occurs on the GDP-bound form of the protein. Phosphorylation of $G_{s\alpha}$ by pp60$^{c\text{-}src}$ was found to increase receptor-induced binding of GTP and GTPase activity. Taken together, these results suggest that one mechanism by which pp60$^{c\text{-}src}$ affects signalling through the β-adrenergic receptor is by phosphorylation and functional alteration of the G protein which links the receptor to adenylate cyclase.

Efforts to extend these studies in intact cells have been hindered by the lack of suitable antibodies capable of immunoprecipitating tyrosine phosphorylated $G_{s\alpha}$ (Hausdorff et al., 1992; J. S. Moyers and S. J. Parsons, unpublished work). To facilitate the design of genetic approaches to this question and to gain insights into the mechanism of pp60$^{c\text{-}src}$ potentiation of β-adrenergic signalling, we undertook the identification of the *in vitro* sites of phosphorylation of $G_{s\alpha}$ by pp60$^{c\text{-}src}$. Two major sites of phosphorylation were mapped, Tyr-37 and Tyr-377. Tyr-37 resides near the N-terminus, proximal to the site of $\beta\gamma$ binding, and Tyr-377 is located at the site of receptor binding in the C-terminus. The locations of these sites suggest that tyrosine phosphorylation of $G_{s\alpha}$ could alter its ability to interact with $\beta\gamma$ subunits and/or the receptor, interactions which could affect guanine nucleotide exchange and GTP hydrolysis. Indeed, such activities of $G_{s\alpha}$ were shown to be altered by pp60$^{c\text{-}src}$ tyrosine phosphorylation in the *in vitro* studies described above.

EXPERIMENTAL

Materials and reagents

Synthetic peptides were obtained from Research Genetics (Huntsville, AL, U.S.A.), AG1-X8 columns were supplied by Bio-Rad, and tosylphenylalanylchloromethane (TPCK)-treated trypsin was purchased from Worthington. The bicinchoninic acid (BCA) protein assay kit was a product of Pierce, Pansorbin was obtained from Calbiochem, and γ-[^{32}P]ATP (6000 Ci/mmol) was supplied by New England Nuclear. X-Omat AR film, cyanogen bromide, all detergents (except Triton X-100) and protease and phosphatase inhibitors were purchased from Sigma. Triton X-100 was obtained from EM Science.

Purification of $G_{s\alpha}$

The short form of recombinant bovine $G_{s\alpha}$ ($G_{s\alpha s}$) was expressed in *Escherichia coli* and purified as described (Graziano et al., 1989). In some experiments, a hexahistidine-tagged form of the protein ($HisG_{s\alpha s}$) was used. A plasmid encoding $G_{s\alpha s}$ with six histidine residues added at the N-terminus was constructed as described (Linder et al., 1993). Vector NpT7-5/$HisG_{s\alpha s}$ was introduced into the BL21-DE3 strain of *E. coli* and $HisG_{s\alpha s}$ was purified according to Lee et al. (1994). Recombinant $G_{s\alpha s}$ and $HisG_{s\alpha s}$ purified from *E. coli* are indistinguishable with respect to interactions with adenylate cyclase and guanine nucleotides (M. E. Linder, unpublished work) and generate the same phosphopeptide map (results not shown). Henceforth in this report $G_{s\alpha s}$ is referred to as $G_{s\alpha}$.

Preparation of pp60$^{c\text{-}src}$

pp60$^{c\text{-}src}$ was immunoprecipitated from a C3H10T$\frac{1}{2}$ mouse fibroblast cell line (5Hd47) which overexpresses avian c-*src* at levels approx. 30-fold above endogenous amounts (Wilson et al., 1989). Immunoprecipitations of pp60$^{c\text{-}src}$ were prepared as described previously (Wilson et al., 1989; Moyers et al., 1993). Briefly, cells were lysed in RIPA buffer [150 mM NaCl, 1% deoxycholate (DOC), 1% Triton X-100, 0.1% SDS and 50 mM Tris (pH 7.2)] with the inhibitors, vanadate (100 μM), aprotinin (1%), leupeptin (50 μg/ml), microcystin (1 μM), and phenylmethanesulphonyl fluoride (1 mM). Extracts were clarified at 10000 *g* for 10 min at 4 °C, and protein concentrations were determined using the BCA assay. Lysates (250 μg) were incubated on ice for 1 h with 2 μg of purified avian pp60$^{c\text{-}src}$-specific monoclonal antibody (mAb), EC10, or a mixture of EC10 and mAb GD11, which recognizes both avian and rodent pp60$^{c\text{-}src}$ (Parsons et al., 1984). Antibodies were adsorbed to Pansorbin for an additional 30 min. Immune complexes were washed once each in HS buffer [1 M NaCl, 0.1% Nonidet P-40 (NP-40), 50 mM Tris, pH 7.2], HO buffer (100 mM NaCl, 1% NP-40, 0.5% DOC, 1 mM EDTA, 50 mM Tris, pH 7.2), and HBS (150 mM NaCl, 20 mM Hepes, pH 7.2). Pp60$^{c\text{-}src}$, purified from baculovirus-infected Sf9 cells, was a generous gift from Dr. Michael J. Weber, University of Virginia.

Kinase assays

For phosphorylation reactions, purified, recombinant $G_{s\alpha}$ (2–5 pmol) was added to immunoprecipitates of pp60$^{c\text{-}src}$ in 30 μl of kinase buffer containing 20 mM Pipes [piperazine-*N*,*N*'-bis(2-ethanesulphonic acid)], 10 mM $MnCl_2$, and 1 μM (10 μCi) [γ-^{32}P]ATP. Reactions were incubated for 30 min at 30 °C and then terminated by the addition of Laemmli sample buffer. Products were separated on SDS/10% polyacrylamide gels. In preparation for phosphotryptic or h.p.l.c. analysis, dried or wet gels were exposed to X-Omat AR film with intensifying screens, and the band corresponding to $G_{s\alpha}$ was excised and eluted from the gel as follows: gel pieces were ground into a fine paste, and protein was eluted in buffer containing 50 mM Hepes, pH 8.0, 0.1% SDS and 0.5% dithiothreitol by overnight incubation at 4 °C on a rotator. Eluted protein was precipitated with trichloroacetic acid at a final concentration of 10% (w/v), washed with deionized

distilled water, and lyophilized in a Savant Speed Vac concentrator.

Two-dimensional phosphotryptic analysis

Trypsin digestion of phosphorylated $G_{s\alpha}$ was carried out according to the method of Boyle et al. (1991). Eluted, lyophilized protein was oxidized with performic acid and subjected to trypsin digestion by the addition of 10 μg of TPCK-treated trypsin twice over a period of 6 h at 37 °C, unless otherwise noted. Phosphotryptic peptide analysis was performed on t.l.c. plates (10 cm × 10 cm) using pH 1.9 buffer (formic acid/acetic acid/water; 5:15.6:179.4, by vol.) for electrophoresis in the first dimension. Following chromatography in the second dimension in buffer containing n-butanol/pyridine/acetic acid/water (15:10:3:12, by vol.), plates were exposed to film for 1 to 2 days.

Phosphoamino acid analysis

Phosphoamino acid analysis of $G_{s\alpha}$ was performed as described by Boyle et al. (1991) on *in vitro* ^{32}P-labelled protein eluted from gels. Samples were hydrolysed in 100 μl of 6 M HCl for 60 min at 110 °C, lyophilized, and resuspended in pH 1.9 buffer containing 1 μg each of phosphoserine, phosphothreonine and phosphotyrosine. Aliquots were analysed by two-dimensional electrophoresis, using pH 1.9 buffer (described above) in the first dimension and pH 3.5 buffer (acetic acid/pyridine/water; 10:1:189, by vol.) in the second dimension. Unlabelled phosphoamino acid standards were visualized by spraying plates with 0.25% (w/v) ninhydrin in acetone. Exposure times ranged from 2 to 3 days.

H.p.l.c. analysis

For h.p.l.c. analysis of $G_{s\alpha}$, ^{32}P-labelled phosphotryptic peptides were prepared as above and suspended in 0.05% trifluoroacetic acid (TFA). Peptides were injected into a Perkin–Elmer Series 4 liquid chromatograph equipped with a Vydac C_{18} column (4.6 mm × 250 mm) and eluted with increasing concentrations of acetonitrile (0 to 100%) at a flow rate of 1 ml/min, as described by Wasilenko et al. (1991). Fractions (500 μl) were collected and Cerenkov counts of each fraction were determined. Samples containing peak levels of radioactivity were lyophilized in preparation for two-dimensional phosphotryptic analysis (see above) or for Edman degradation (see below).

Edman degradation

Fractions of ^{32}P-labelled $G_{s\alpha}$ peptides, separated by h.p.l.c., were subjected to automated Edman degradation performed by the University of Virginia Biomolecular Research Facility. Phosphorylated peptides were coupled to a Sequelon aryl amine membrane (Coull et al., 1991), washed four times with 1 ml aliquots of 27% acetonitrile/9% TFA and twice with 1 ml aliquots of 50% methanol, and transferred to an Applied Biosystems 470A sequenator using the cartridge inverted as suggested by Stokoe et al. (1992). The cycle used for sequencing was based on that of Meyer et al. (1991), but modified by direct collection of anilinothiazolinone amino acids in TFA as described by Russo et al. (1992). Radioactivity was measured by Cerenkov counting.

Phosphorylation of synthetic peptides

For phosphorylation reactions, 10 μg of purified synthetic peptide, corresponding to either the $G_{s\alpha}$ tryptic fragment, ^{33}DKQVYR^{38}, or ^{376}QYELL^{380}, was added to immune-complexed $pp60^{c\text{-}src}$. Kinase reactions were performed as above, except that incubation at 30 °C was carried out for up to 6 h. Following incubation, reactions were centrifuged at 10000 *g* for 10 min to pellet the $pp60^{c\text{-}src}$ immune complexes. Free [γ-^{32}P]ATP was removed by ion-exchange chromatography as described by Kemp et al. (1976). Briefly, supernatants containing the phosphopeptides were applied to AG1-X8 columns that had been equilibrated with 30% acetic acid. Phosphopeptides were eluted with 30% acetic acid, and phosphotryptic analysis was performed as above.

RESULTS

Phosphorylation of $G_{s\alpha}$ by $pp60^{c\text{-}src}$

Hausdorff et al. (1992) have shown that immune-complexed $pp60^{c\text{-}src}$ phosphorylates purified recombinant G_{α} on tyrosine residues. Figure 1(a) shows the results of an *in vitro* kinase assay in which purified recombinant bovine $G_{s\alpha}$ was phosphorylated by $pp60^{c\text{-}src}$ immunoprecipitated from C3H10T$\frac{1}{2}$ cells which overexpress c-*src*. We routinely achieved stoichiometries of phosphorylation ranging from 0.4–0.9 mol of PO_4/mol of substrate. Phosphorylation occurred on tyrosine residues as assessed by phosphoamino acid analysis (Figure 1b). $pp60^{c\text{-}src}$ purified from baculovirus-infected Sf9 cells was also found to phosphorylate $G_{s\alpha}$ to stoichiometries similar to that for immune-complexed $pp60^{c\text{-}src}$ (results not shown).

H.p.l.c. analysis of $G_{s\alpha}$ tryptic peptides

The protein sequence of bovine $G_{s\alpha}$ (Robishaw et al., 1986) reveals that the molecule contains 14 tyrosine residues contained within 11 of the 54 potential tryptic fragments. To isolate tryptic peptides containing phosphorylated tyrosine residues, phosphorylated $G_{s\alpha}$ was eluted from gels and subjected to trypsin digestion. The resulting phosphopeptides were applied to an h.p.l.c. column and separated by elution with increasing concentrations of acetonitrile. Figure 2 shows the resulting h.p.l.c. profile of the eluted fractions, where three major peaks of radioactivity were obtained (Figure 2). These fractions were subjected to two-dimensional phosphotryptic analysis as shown

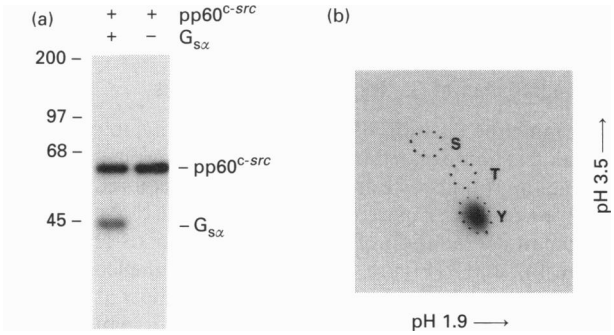

Figure 1 ***In vitro* phosphorylation of $G_{s\alpha}$ by $pp60^{c\text{-}src}$**

Purified recombinant bovine $G_{s\alpha}$ (500 ng) was incubated with immune-complexed $pp60^{c\text{-}src}$ (prepared from 250 μg of lysate of 5Hd47 cells) in a phosphorylation reaction as described in the Experimental section. Products were analysed by SDS/PAGE on a 10% gel and visualized by autoradiography (**a**). Numbers to the left denote the migration of the molecular-mass standards myosin (200 kDa), phosphorylase B (97 kDa), BSA (68 kDa), and ovalbumin (45 kDa). The band corresponding to $G_{s\alpha}$ was excised and subjected to phosphoamino acid analysis (**b**). The migration of phosphoamino acid standards is outlined.

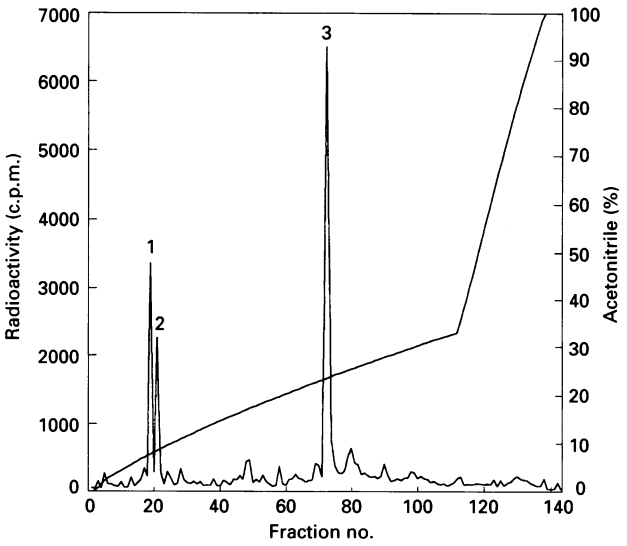

Figure 2 Separation of $G_{s\alpha}$ phosphopeptides by h.p.l.c.

Recombinant $G_{s\alpha}$ was phosphorylated *in vitro* by $pp60^{c\text{-}src}$ using [γ-^{32}P]ATP, eluted from a 10% polyacrylamide gel and subjected to trypsinization as described in the Experimental section. The resulting peptides were separated by h.p.l.c. with increasing concentrations of acetonitrile. Cerenkov counts of each fraction are represented.

in Figure 3. Figure 3(a) shows the phosphotryptic pattern of the total $G_{s\alpha}$ peptides which were loaded on to the h.p.l.c. column. Figures 3(b), 3(d) and 3(f) show the location on the t.l.c. plate of phosphotryptic fragments from peaks 1, 2 and 3 respectively. In order to determine the location of these peptides in the profile of total $G_{s\alpha}$ peptides, a sample of each of the h.p.l.c. peak fractions was mixed with a portion of the total $G_{s\alpha}$ phosphopeptides and analysed on t.l.c. plates. Figures 3(c), 3(e) and 3(g) are the patterns resulting from mixing portions of peak fractions 1, 2 or 3 respectively, with the whole phosphotryptic sample. The phosphorylated peptides in peak fractions 1, 2 and 3 were localized to the positions designated 1, 2 and 3 on the chromatograms respectively, where the intensity was more than doubled when the samples were mixed. The phosphotryptic pattern of $G_{s\alpha}$, phosphorylated by $pp60^{c\text{-}src}$ purified from baculovirus, was nearly identical with that depicted in Figure 3(a) (results not shown).

Edman degradation of $G_{s\alpha}$ tryptic phosphopeptides

To determine the position of the phosphotyrosine in peptides of each peak fraction isolated by h.p.l.c., fractions were analysed to determine the rounds of Edman degradation required to release the labelled residue. Figure 4 shows the radioactivity recovered from Edman degradation of peptides from peaks 1 (Figure 4a), 2 (Figure 4b), and 3 (Figure 4c). Peptide 1 was labelled at residue 3, peptide 2 was labelled at residue 5 and peptide 3 at residue 2. Table 1 shows the predicted tryptic peptides of $G_{s\alpha}$ which contain the 14 tyrosine residues present in the protein. Three tyrosines are located at position three of the tryptic peptides, Tyr-37, Tyr-116 and Tyr-149. While no tyrosine residues are located five amino acids C-terminal to a site of trypsin cleavage, Tyr-37 is located three amino acids from a site of trypsin cleavage at Arg-35 in the peptide, QVY^{37}R, and five amino acids from a site of cleavage at Arg-33 in the peptide, DKQVY^{37}R. No other tyrosine residue is positioned in such a manner. To determine whether the peptide in h.p.l.c. peak 1 is derived from the peptide in peak 2,

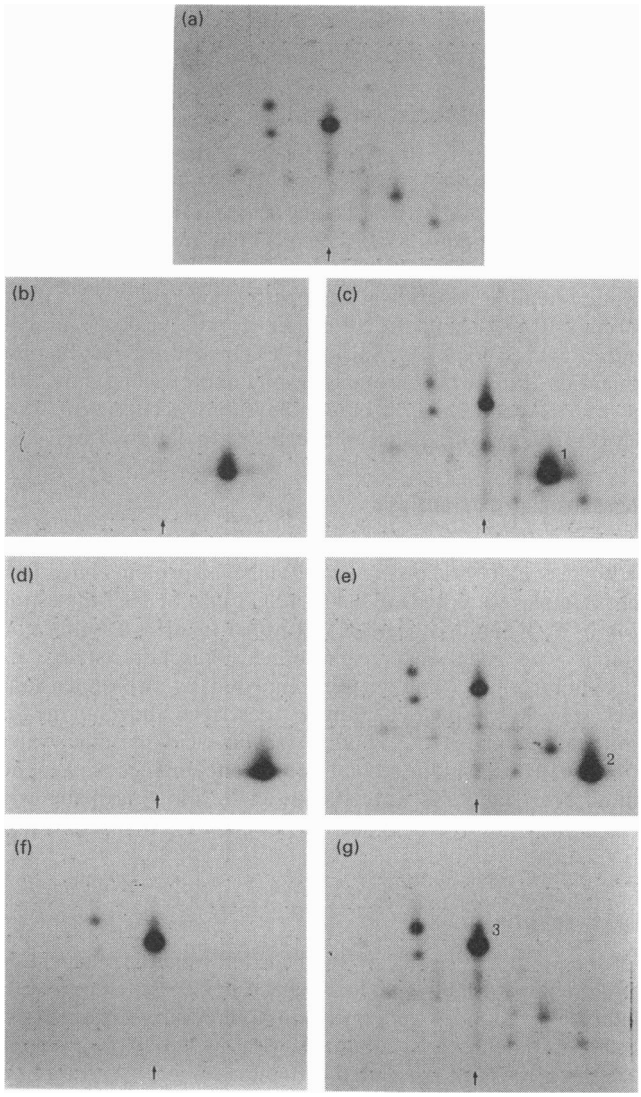

Figure 3 T.l.c. analysis of h.p.l.c.-purified $G_{s\alpha}$ tryptic phosphopeptides

H.p.l.c.-purified phosphotryptic peptides of $G_{s\alpha}$ were analysed by two-dimensional electrophoresis and chromatography on t.l.c. plates as described in the Experimental section. (**a**) Map of total tryptic phosphopeptides of $G_{s\alpha}$ derived from the starting material for h.p.l.c. analysis. (**b**), (**d**) and (**f**), T.l.c. profiles of h.p.l.c. peaks 1, 2 and 3 respectively. (**c**), (**e**) and (**g**), mixes of h.p.l.c. peaks 1, 2 and 3 respectively, with the total $G_{s\alpha}$ phosphotryptic sample depicted in (**a**). 200 Cerenkov c.p.m. of each sample were loaded on to t.l.c. plates. Mixes contained 200 c.p.m. of h.p.l.c.-purified peptide and 200 c.p.m. of total sample before h.p.l.c. purification. Plates were exposed to film for 1 day at −70 °C with intensifying screens.

a portion of the $G_{s\alpha}$ peptides used as starting material for h.p.l.c. analysis was subjected to trypsin digestion for an additional 18 h in an effort to achieve more complete digestion. Indeed, prolonged exposure to trypsin resulted in a nearly complete depletion of the phosphotryptic spot corresponding to peptide 2, and a concomitant increase in intensity of the spot corresponding to peptide 1 (results not shown). These findings suggested that peptide 2 is an incomplete digestion product containing peptide 1 and that both peptides contain phosphorylated Tyr-37.

Table 1 reveals that two tyrosines are located two residues C-terminal to a site of trypsin cleavage. These are Tyr-344, which resides in the peptide, HY^{344}CYPHSTCAVDTENIR, and Tyr-377, contained within the peptide, QY^{377}ELL. While Tyr-377 is

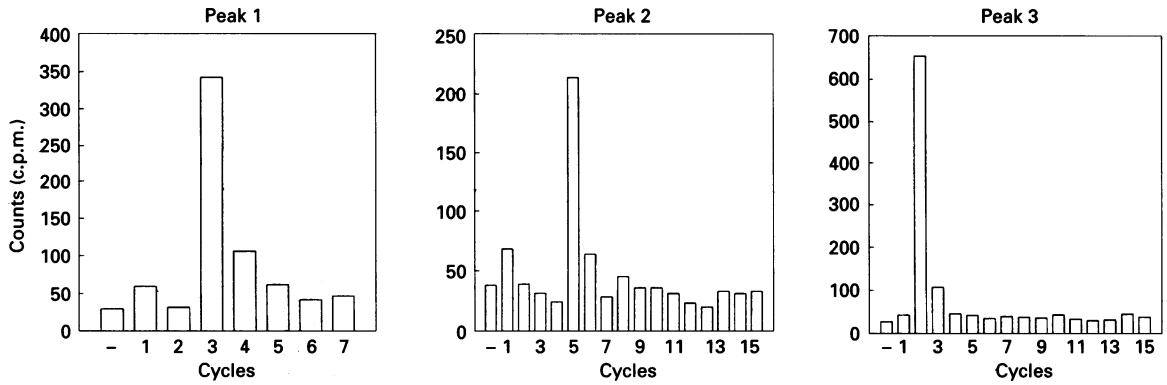

Figure 4 Edman degradation of $G_{s\alpha}$ tryptic phosphopeptides

H.p.l.c.-purified phosphopeptides were subjected to sequential rounds of Edman degradation, as described in the Experimental section, to release the phosphorylated residues. (**a**), (**b**) and (**c**) show c.p.m. values recovered from analysis of h.p.l.c. peak fractions 1, 2 and 3 respectively. The starting material for analysis was as follows: peak 1, 1800 c.p.m.; peak 2, 980 c.p.m.; peak 3, 4600 c.p.m. Cerenkov counts of fractions obtained from each round of degradation are shown. In each panel the first bar represents an unlabelled blank control. Cycle number for degradation of labelled peptides begins with the second bar.

Table 1 Location of tyrosine residues in predicted tryptic peptides of $G_{s\alpha}$*

Tyrosine residues are indicated in bold type.

Peptide	Sequence
1	QV**Y**^{37}R (contained within the incomplete tryptic peptide, DKQV**Y**^{37}R)
2	VD**Y**^{116}ILSVMNVPDFDFPPEF**Y**^{133}EHAK
3	AC**Y**^{149}ER
4	SNE**Y**^{155}QLIDCAQ**Y**^{163}FLDK
5	QDD**Y**^{176}VPSDQDLLR
6	WIQCFNDVTAIIFVVASSS**Y**^{239}NMVIR
7	IED**Y**^{297}FPEFAR
8	**Y**^{304}TTPEDATPEPGEDPR
9	**Y**^{325}FIR
10	H**Y**^{344}C**Y**^{346}PHSTCAVDTENIR
11	Q**Y**^{377}ELL

* Sequence based on Robishaw et al. (1986).

found within a tryptic peptide of only five amino acids, the predicted chromatographic mobility of this fragment is greater than that of the 17-amino-acid fragment containing residue Tyr-344 (calculations based on the method of Boyle et al., 1991), consistent with the migration of this fragment in the chromatographic dimension on t.l.c. plates. In addition, it has been observed that the $G_{s\alpha}$ antibody, RM/1, raised against a peptide encompassing this tryptic fragment (residues RMHLRQY^{377} ELL; Simonds et al., 1989), is incapable of efficiently recognizing the phosphorylated form of $G_{s\alpha}$ (Hausdorff et al., 1992; J. S. Moyers and S. J. Parsons, unpublished work), further supporting the localization of the phosphate to Tyr-377 and suggesting that phosphorylation of this residue affects the recognition of this epitope by the antibody.

Phosphorylation of synthesized peptides

To confirm that Tyr-37 and Tyr-377 were substrates for phosphorylation by $pp60^{c\text{-}src}$, we tested the ability of synthetic peptides containing these residues to be phosphorylated during *in vitro* kinase reactions and to co-migrate on t.l.c. plates with the tryptic phosphopeptides 1, 2 and 3, generated from intact $G_{s\alpha}$. Samples (10 μg) of purified synthetic peptides were incubated for up to 6 h in a kinase reaction with immune-complexed $pp60^{c\text{-}src}$, as described in the Experimental section. Products were purified by ion-exchange chromatography and analysed by separation on t.l.c. plates and autoradiography. Figure 5(a) shows the mobility of trypsin-digested $G_{s\alpha}$ used in mixes with the phosphorylated synthetic peptides. Figure 5(b) shows the mobility of the phosphorylated peptide, DKQVYR, the tryptic peptide containing Tyr-37 at the fifth position and a site of trypsin cleavage following the second amino acid. A mixture of this peptide with trypsin-digested, phosphorylated $G_{s\alpha}$ yielded a pattern that exhibited co-migration of this peptide with the phosphopeptide obtained in the peak 2 h.p.l.c. fraction (Figure 5c). To determine whether trypsin digestion of this material would yield the peptide present in the peak 1 h.p.l.c. fraction, the phosphorylated synthetic peptide was digested with trypsin for 6 h prior to two-dimensional peptide analysis. Figure 5(d) shows that peptide 1 was generated from peptide 2 by trypsin cleavage. These results confirmed that $G_{s\alpha}$ is phosphorylated on Tyr-37.

The synthetic peptide, QYELL, which contains Tyr-377 at the second position, was phosphorylated by immune-complexed $pp60^{c\text{-}src}$ and subjected to two-dimensional phosphotryptic analysis. Figure 5(e) shows the migration of this peptide on t.l.c. plates. T.l.c. analysis of this same material following overnight storage at 4 °C indicated that the peptide was readily altered, yielding the additional fragment shown in Figure 5(f). The migration of this fragment was consistent with the loss of a positive charge, possibly by cyclization of the N-terminal glutamine (Q). Figure 5(g) depicts the results of mixing this material with total peptides of trypsin-digested $G_{s\alpha}$. Phosphopeptide QYELL co-migrated with the peptide which was isolated in the peak 3 h.p.l.c. fraction. The degradation product of this peptide co-migrated with a fragment which was observed in t.l.c. analysis of the peak 3 h.p.l.c. fraction (Figure 3f). Together, these results showed that recombinant bovine $G_{s\alpha}$ is phosphorylated by $pp60^{c\text{-}src}$ on Tyr-37 and Tyr-377.

DISCUSSION

We report here that $pp60^{c\text{-}src}$ phosphorylates $G_{s\alpha}$ on Tyr-37 and Tyr-377 *in vitro*. Tyr-37 of $G_{s\alpha}$ lies near a domain within the molecule that mediates $\beta\gamma$ binding (Johnson et al., 1991), while

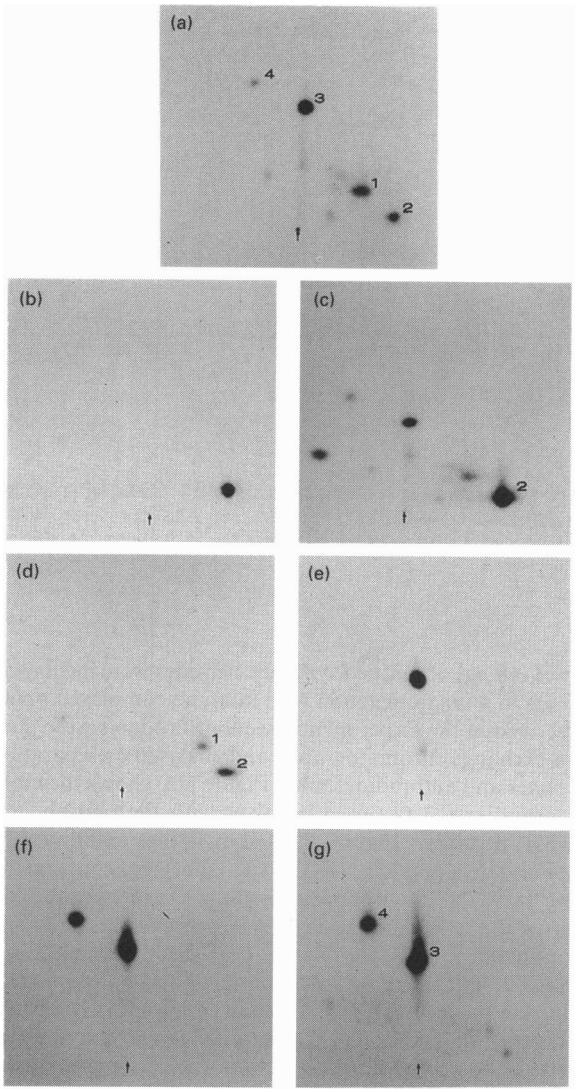

Figure 5 Co-migration of phosphorylated synthetic peptides of $G_{s\alpha}$ with $G_{s\alpha}$ phosphotryptic peptides

Synthetic peptides corresponding to the tryptic fragments DKQVYR and QYELL, containing the predicted sites of phosphorylation of $G_{s\alpha}$ by $pp60^{c\text{-}src}$, were subjected to phosphorylation in an *in vitro* kinase assay and analysis by two-dimensional electrophoresis and chromatography as described in the Experimental section. (**a**) Total $G_{s\alpha}$ tryptic peptides; (**b**), *in vitro* phosphorylated peptide DKQVYR; (**c**), mixture of phosphopeptide DKQVYR with total $G_{s\alpha}$ tryptic phosphopeptides; (**d**), phosphopeptide DKQVYR following 6 h trypsinization; (**e**), *in vitro* phosphorylated peptide QYELL; (**f**), re-analysis of peptide QYELL after 24 h storage at −20 °C; (**g**), mixture of stored phosphopeptide QYELL with $G_{s\alpha}$ phosphotryptic peptides. The spot migrating closest to the cathode in several panels represents free $^{32}P_i$. 1000 Cerenkov counts of each sample was applied to t.l.c. plates. Exposure time was 12 h at −70 °C.

Tyr-377 abuts the receptor interaction domain (Sullivan et al., 1987). Both phosphorylation sites are thus ideally positioned to influence critical aspects of $G_{s\alpha}$ regulation.

Tyr-37 is contained within the attenuator region of $G_{s\alpha}$ that spans residues 15 to 144. Through mutational analysis this region has been postulated to regulate the rate of guanine nucleotide exchange (Johnson et al., 1991). The location of Tyr-37 in this region suggests that phosphorylation of Tyr-37 may be responsible for the alterations in functional activation of the protein, as previously described by Hausdorff et al. (1992). In this report phosphorylation of $G_{s\alpha}$ *in vitro* by $pp60^{c\text{-}src}$ was shown to result in an increased rate of GTP[S] binding. Furthermore, phosphorylation appeared to be inhibited by the presence of $\beta\gamma$ subunits, suggesting that the binding of $\beta\gamma$ sterically hinders phosphorylation or that conformational changes induced by $\beta\gamma$ binding render $G_{s\alpha}$ a less efficient substrate for $pp60^{c\text{-}src}$. In either case, it is possible that phosphorylation of Tyr-37 may affect the interaction of α and $\beta\gamma$ subunits. Reduced levels or affinity of $\beta\gamma$ association are consistent with more rapid nucleotide exchange. In support of this hypothesis is the finding by Lounsbury et al. (1993) that $G_{z\alpha}$ is phosphorylated by protein kinase C on Ser-27 (major) and Ser-25 (minor), residues which are located at the sites of $\beta\gamma$ binding. Phosphorylation of these residues *in vitro* appears to affect the ability of $G_{z\alpha}$ to interact with $\beta\gamma$ subunits.

Tyr-377 lies in a region of $G_{s\alpha}$ required for interaction with the β-adrenergic receptor (Sullivan et al., 1987; Johnson et al., 1991). This finding suggests that phosphorylation of Tyr-377 may affect the association of these two molecules, modulating receptor-mediated dissociation of GDP and subsequent activation of $G_{s\alpha}$. Analysis of the crystal structure of transducin provides some insights into this notion.

The crystal structure of transducin-α complexed with GTP[S] depicts a structure which consists of two domains flanking a guanine-nucleotide-binding core (Noel et al., 1993). One domain, the GTPase domain, is a structure with striking similarity to $p21^{Ha\text{-}ras}$, while the other domain, a highly helical structure, is unique to heterotrimeric G proteins. This domain and the GTPase domain, connected by linker sequences, completely encase the GTP[S] molecule with the helical domain, appearing to form an enclosing lid. This overall structure suggests that a conformational change that results in an opening of this lid is necessary in order to expose the guanine nucleotide cleft during the process of nucleotide exchange. While the crystal structure of $G_{t\alpha}$ lacks the N-terminal 25 residues of the molecule which comprise the binding site for $\beta\gamma$, it is clear that the residues corresponding to Tyr-37 and Tyr-377 reside near each other and proximal to the GTPase domain. Tyr-377 lies in a portion of the molecule which directly contacts a 'hinge' region (the $\alpha 2/\beta 4$ loop that contains residues 211–215 of $G_{t\alpha}$), a domain which interacts with the terminal phosphate of GTP. It is interesting to speculate that phosphorylation of this region could enhance receptor-mediated conformational changes which regulate the opening of the nucleotide-binding cleft and lead to dissociation of GDP. Such a scenario would provide an explanation for the enhancement in receptor-mediated dissociation of GDP and subsequent binding of GTP[S] observed in phosphorylated $G_{s\alpha}$ (Hausdorff et al., 1992). Additionally, phosphorylation of Tyr-377 could facilitate GTPase activity by altering conformational parameters regulating hydrolysis of the γ-phosphate of GTP.

In addition to phosphorylating $G_{s\alpha}$, $pp60^{c\text{-}src}$ is capable of phosphorylating other G_α subtypes on tyrosine residues *in vitro* (Hausdorff et al., 1992). However, it has not yet been determined whether $pp60^{c\text{-}src}$ can participate in signalling through pathways coupled to these molecules. We were surprised to find that neither of the major sites of $G_{s\alpha}$ tyrosine phosphorylation were conserved among most other G_α subunits (Birnbaumer et al., 1990; GenBank). Exceptions were G_{olf}, which has the QYELL motif and an analogous Y^{37} (ERLAYK versus DKQVYR), $G_{q\alpha}$, which contains a Tyr-377 homologue, and $G_{z\alpha}$, which contains a tyrosine one residue upstream of this position. Neither $G_{q\alpha}$ nor $G_{z\alpha}$ have counterparts to Tyr-37. However, $G_{z\alpha}$ contains two serines in close proximity to Tyr-37 (Ser-25 and Ser-27), which serve as sites of phosphorylation by protein kinase C and may function analogously to Tyr-37 (Lounsbury et al., 1993). The high degree of sequence similarity between $G_{s\alpha}$ and G_{olf} (approx. 90 % identical) suggests that these two proteins may be subject to

similar regulatory mechanisms. *In vitro* phosphorylation of other G_{α} proteins by $pp60^{c\text{-}src}$ must occur on alternative residues. Whether these sites are located in similar functional regions is an issue that requires further exploration.

Finally, while *in vivo* phosphorylation of $G_{s\alpha}$ on tyrosines 37 and 377 has yet to be conclusively demonstrated, several lines of evidence nevertheless suggest that the *in vivo* function of $G_{s\alpha}$ is influenced by $pp60^{c\text{-}src}$. First, overexpression of $pp60^{c\text{-}src}$ potentiates both agonist-induced and cholera toxin-induced cyclic AMP accumulation in intact cells (Bushman et al., 1990; Moyers et al., 1993). Secondly, $G_{s\alpha}$ can be precipitated from these cells using phosphotyrosine antibodies, suggesting that it may be tyrosine phosphorylated *in vivo* (Hausdorff et al., 1992; J. S. Moyers and S. J. Parsons, unpublished work). Finally, $pp60^{c\text{-}src}$ phosphorylates $G_{s\alpha}$ *in vitro*, resulting in an enhanced rate of GTP binding and GTPase activity of this protein. Numerous attempts to investigate tyrosine phosphorylation of $G_{s\alpha}$ *in vivo* have been hampered by the lack of suitable $G_{s\alpha}$-specific antibodies. Identification of the sites of $G_{s\alpha}$ *in vitro* phosphorylation by $pp60^{c\text{-}src}$ will facilitate the design of genetic approaches to address this question and to determine the effects of modification of these residues on the ability of $G_{s\alpha}$ to mediate signals from receptor to effector.

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