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Thiophosphorylation of the G Protein β Subunit in Human Platelet Membranes: Evidence against a Direct Phosphate Transfer Reaction to G_α Subunits

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SUMMARY

A direct phosphate transfer reaction from the G protein β subunits to either $G_{s\alpha}$ or $G_{i\alpha}$ has been proposed to account for the ability of thiophosphorylated transducin βγ-dimers to bidirectionally regulate adenylyl cyclase activity in human platelet membranes. We searched for experimental evidence for this reaction. Incubation of human platelet membranes with [^{35}S]guanosine-5′-(3-*O*-thio)triphosphate ([^{35}S]GTPγS) results in the predominant incorporation of [^{35}S]thiophosphate into a 36-kDa protein, which comigrates with the G protein β subunit and is immunoprecipitated by a β subunit-specific antiserum. Thiophosphorylation of the β subunit is specific for guanine nucleotides and abolished by the histidine-modifying agent diethylpyrocarbonate and heat and acid treatment. Dephosphorylation of [^{35}S]thiophosphorylated β subunits is accelerated in the presence of GDP, but not ADP, UDP, or guanosine-5′-(2-*O*-thio)diphosphate. Neither the thiophosphorylation nor the dephosphorylation is sensitive to receptor agonists (α_2-adrenergic, A_2 adenosine, thrombin, or insulin), and purified G protein α subunits do not act as thiophosphate donors. An approach was designed to demonstrate direct thiophosphate transfer to protein-bound nucleotides; platelet membranes were sequentially exposed to $NaIO_4$, $NaCNBH_3$, and $NaBH_4$, an oxidation-reduction step that covalently incorporates prebound nucleotides into proteins. Under these conditions, multiple radiolabeled proteins are visualized on subsequent addition of [^{35}S]GTPγS. This reaction is specific because both oxidation and reduction are required and pretreatment of platelet membranes with 2′,3′-dialdehyde GTPγS or diethylpyrocarbonate blocks the subsequent labeling in oxidized and reduced membranes. The G protein β subunit may participate in this thiophosphate transfer reaction. Most important, however, no labeled G protein α subunits ($G_{s\alpha}$ and $G_{i\alpha}$) were recovered by immunoprecipitation from oxidized and reduced membranes subsequent to the addition of [^{35}S]GTPγS. Thus, our results clearly rule out the existence of a postulated G protein activation by phosphate transfer reactions, which lead to the formation of GTP from GDP prebound to the α subunit.

The receptor-catalyzed exchange of prebound GDP for GTP on the G protein α subunit is the reaction that initiates signal transduction by heterotrimeric G proteins (1). Several studies have indicated that an additional ill-defined mechanism involving a transphosphorylation reaction is important for the control of G protein-mediated signal transduction. The experimental evidence can be summarized as follows: (i) UDP, which blocks transphosphorylation of adenine and guanine nucleotides, reverses the ability of guanine nucleotides to inhibit platelet adenylyl cyclase activity (2). (ii) A receptor-dependent transfer of thiophosphate from ATPγS to GDP generates GTPγS from GDP and thus abolishes high affinity binding of agonists to G protein-coupled receptors (3, 4). This mechanism appears to be widely conserved across species because it can also be observed in the slime mold *Dictyostelium discoideum* (5). (iii) In the presence of [^{35}S]GTPγS, a thiophosphorylated transducin β subunit complex is eluted from retinal rod outer segments. When added to cell membranes, the purified thiophosphorylated β subunit is capable of both mediating activation and inhibition of adenylyl cyclase. This has been interpreted as evidence of transfer of the thiophosphate onto G protein α subunits (6). (iv) A NDP-kinase activity coimmunoprecipitates with G protein oligomers (7). Therefore, a hypothesis has been put forth that emphasizes the role of NDP-kinase in G protein-mediated signal transduction (8, 9). Although the precise mechanism is not understood and the function of NDP-kinase is not clear, it

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ABBREVIATIONS: GTPγS, guanosine-5′-(3-*O*-thio)triphosphate; UK 14,304, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine; Gpp(NH)p, guanosine-5′-*O*-(β,γ-imino)triphosphate; PMSF, phenylmethylsulfonyl fluoride; NP-40, Nonidet P40; CHAPS, 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DEPC, diethylpyrocarbonate; NDP-kinase, nucleoside diphosphate kinase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; oGTP, 2′,3′-dialdehyde analogue of GTP.

is worth noting that the product of the human tumor suppressor gene *nm23* is a NDP-kinase (10, 11). Similarly, mutations in the *awd* (abnormal wing disc) gene of *drosophila*, which codes for an NDP-kinase, are associated with developmental defects (12). This has spurred attempts to provide evidence of a direct interaction between NDP-kinase and both heterotrimeric and small G proteins. Efforts have been focused on demonstrating that a direct phosphate transfer reaction exists, which leads to formation of GTP within the guanine nucleotide binding pocket (13–16). Experiments have been subjected to broad criticism and the conclusions have been questioned; specifically, the experimental problem, which could not be circumvented, was the fact that GDP prebound to the protein was released during the reaction and phosphorylated in solution by NDP-kinase (14, 16).

In the present work, we reexamined the possibility that phosphate transfer reaction involving G protein subunits occurs in cell membranes and used an experimental approach in which the nucleotides are covalently attached to potential acceptor proteins. Our observations confirm that the G protein β subunit is subject to thiophosphorylation in the presence of [^{35}S]GTPγS. We also observed subsequent transfer of the thiophosphate to other membrane proteins but found no evidence of participation of G protein α subunits as thiophosphate donors or acceptors in this reaction.

Experimental Procedures

Materials. [^{35}S]GTPγS and [α-^{32}P]ATP were purchased from NEN (Boston, MA). Guanine and adenine nucleotides, adenosine deaminase, and trypsin were obtained from Boehringer Mannheim (Mannheim, Germany); NECA was from RBI (Natick, MA); $NaBH_4$, $NaCNBH_3$, $NaIO_4$, polyethylenimine/cellulose thin layer chromatography plates, buffers, and salts were from Merck (Darmstadt, Germany), and DEPC was from Fluka (Buchs, CH). Protein standards for electrophoresis covering the range of 14–100 kDa were from Bio-Rad (Richmond, CA). NP-40, Tween 20, sodium deoxycholate, thrombin, insulin, and UK 14,304 were obtained from Sigma Chemical Co. (St. Louis, MO), and aprotinin was from Bayer (Wuppertal, Germany). Nitrocellulose membranes and filters were obtained from Schleicher & Schuell. The sources of the materials required for G protein purification have been described previously (17, 18). oGTPγS and oGTP were synthesized as described (19). The antisera that were used (20) were antiserum B2N (raised against the peptide corresponding to residues 25–39 of the β2 subunit) and AS7 (raised against the carboxyl-terminal decapeptide of $G_{t\alpha}$) (both were the generous gifts of G. Milligan, Department of Biochemistry, Glasgow University, U.K.). Antiserum 7 corresponds to the original antiserum K521 and was raised against a peptide comprising residues 8–23 in the sequence of the β_1/β_2 subunit. Antiserum 333 corresponds to antiserum 584 and was raised against the amino acids 311–325 in $G_{s\alpha\text{-}s}$ (unique to $G_{s\alpha}$).

Protein purification and membrane preparation. $rG_{s\alpha\text{-}L}$ and $rG_{s\alpha\text{-}s}$ (the long and short splice variants of the recombinant α subunit of the G_s that stimulates adenylyl cyclase) $rG_{i\alpha\text{-}1}$, and $rG_{i\alpha\text{-}2}$ ($rG_{i\alpha}$ is the recombinant myristoylated G protein α subunit of G_i, which mediates inhibition of adenylyl cyclase) were purified from bacterial lysates (17, 18). G protein $\beta\gamma$-dimers were chromatographically resolved from the $G_{o,i}$ oligomers (21). Human platelet membranes were prepared as previously described (22).

Modification of platelet membranes by sequential oxidation and reduction. Sequential oxidation and reduction was achieved according to the procedure of Löw *et al.* (23) with minor modifications. Briefly, for *in situ* oxidation, platelet membranes were diluted in an HEM buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid·NaOH, pH 7.6, 2 mM $MgSO_4$, 1 mM EDTA) to a protein concentration of ~1–1.5 mg/ml and incubated with 4 mM $NaIO_4$ for 1 min at 22°. The oxidation was subsequently stopped by the addition of 80 mM $NaCNBH_3$ (1 min at 22°), followed by 40 mM $NaBH_4$ (30 min on ice) to achieve complete reduction of any free oxidized nucleotide. The suspension was centrifuged twice (50,000 × *g* for 15 min at 2°) and resuspended in TEN buffer (50 mM triethanolamin·HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl). The treated membranes are referred to as oxidized and reduced membranes.

Thiophosphorylation of platelet membranes. Untreated (native) platelet membranes or oxidized and reduced platelet membranes (1–2 mg/ml) were incubated in TEMN buffer (50 mM triethanolamin·HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 2 mM $MgCl_2$) at 22° in 20–40 μl. The reaction was started by the addition of 0.1 μM [^{35}S]GTPγS (~500 cpm/fmol) and was stopped with Laemmli sample buffer supplemented with 40 mM DTT. Alternatively, in experiments, in which buffer exchange was required, the mixture was centrifuged at 50,000 × *g* for 5 min at 4°.

Adenylyl cyclase determinations. Adenylyl cyclase activity in human platelet membranes was assayed in the absence of an ATP-regenerating system as described in Ref. 2 with the following modifications: platelet membranes (5–7 μg) were incubated in 15 μl containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid·NaOH, pH 7.6, 5 mM $MgCl_2$, and 1 mM EDTA in the absence and presence of 10 μM oGTP for 5 min at 20° followed by the addition of the same solution (80 μl, prewarmed to 20°) containing the addition of 50 μM ATP, 50 μM forskolin, and concentrations of nucleotides and the α_2-adrenergic agonist UK 14,304 (as indicated in Fig. 6 legend). After 5 min at 20°, 5 μl [α-^{32}P]ATP (1 μCi) was added, and the incubation continued for an additional 10 min. Under these conditions, forskolin-stimulated activity was linear with regard to time and added platelet membrane protein as previously documented by Jakobs and Wieland (2). The cAMP formed was purified by double-column chromatography (24).

Immunoprecipitation and immunoblots. Human platelet membranes (250–500 μg) were thiophosphorylated in TEM buffer containing 10 mM $MgSO_4$ and 100 nM [^{35}S]GTPγS (for specific activity, see above) for 5 min at 22° in a volume of 250 μl. The TEMN buffer was removed by centrifugation (50,000 × *g* for 15 min) and the pelleted membranes were resuspended in 250 μl denaturation buffer (10 mM Tris·HCl, pH 8, 25 mM EDTA, 0.8% SDS, 0.2 mM PMSF). After 30 min, the insoluble material was removed by centrifugation at 50,000 × *g* for 30 min. The supernatant was supplemented with 8 volumes of immunoprecipitation buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 25 mM EDTA, 1 mM DTT, 1 mM NaF, 1% NP-40, 1% sodium-deoxycholate, 0.2 mM PMSF, 0.003 mM aprotinin, 0.01 mM GTPγS), precleared with pansorbin or protein A-conjugated Sepharose beads, and incubated under continuous stirring with 5 μl of antiserum 333 (specific for $G_{s\alpha\text{-}s}$ and $G_{s\alpha\text{-}L}$), 5 μl antiserum AS7 (specific for $G_{i\alpha\text{-}1}$ and $G_{i\alpha\text{-}2}$), or 5 μl antiserum 7 (specific for the G protein β subunit) at 4°. After 3–4 hr, 10 μl of 10% pansorbin was added, and the mixture was incubated for an additional 2 hr; alternatively, protein A-conjugated Sepharose beads were used. The incubation was centrifuged at 13,000 × *g* for 5 min. The resulting pellet was washed five times with RIPA buffer (1% NP-40, 50 mM Tris·HCl, pH 8.3, 5 mM EDTA, 150 mM NaCl), subsequently resuspended in 25 μl Laemmli sample buffer supplemented with 40 mM DTT, and heated at 50° for 5 min. The supernatant was subjected to SDS-polyacrylamide gel electrophoresis. $rG_{s\alpha\text{-}s}$, $rG_{s\alpha\text{-}L}$, and $rG_{i\alpha\text{-}1}$ were radiolabeled with [^{35}S]GTPγS with the use of *in situ* periodate oxidation as described previously (19), and they were used as molecular mass standards.

For immunoblots, proteins resolved on SDS-polyacrylamide gels were transferred electrophoretically to nitrocellulose membranes. Nitrocellulose sheets were incubated sequentially with 1% bovine serum albumin in 20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, and the same solution containing the appropriate antisera at a dilution of 1:1000. Immunoreactive bands were visualized using

a second antibody conjugated to alkaline phosphatase (Promega, Madison, WI) or to horseradish peroxidase (Amersham, Amersham, UK). Each experiment was reproduced at least three times.

Results

The addition of [^{35}S]GTPγS to human platelet membranes leads to the predominant incorporation of radioactivity into a ~36-kDa protein (Fig. 1A). An increase in the magnesium concentration to 50 mM results in the labeling of additional proteins, which are, however, much less prominent than the 36-kDa protein. This [^{35}S]thiophosphorylated 36-kDa band comigrates with the immunoreactivity detected with G protein β subunit-specific antiserum B2N and the purified β subunit (Fig. 1B). Therefore, the G protein β subunit is the most likely candidate acceptor protein for thiophosphorylation. We corroborated this interpretation by immunoprecipitating the ^{35}S-labeled protein with antiserum 7, a second antiserum directed against a different sequence of the G_β subunit (Fig. 1C). The immunoprecipitated protein was again recognized by antiserum B2N (not shown).

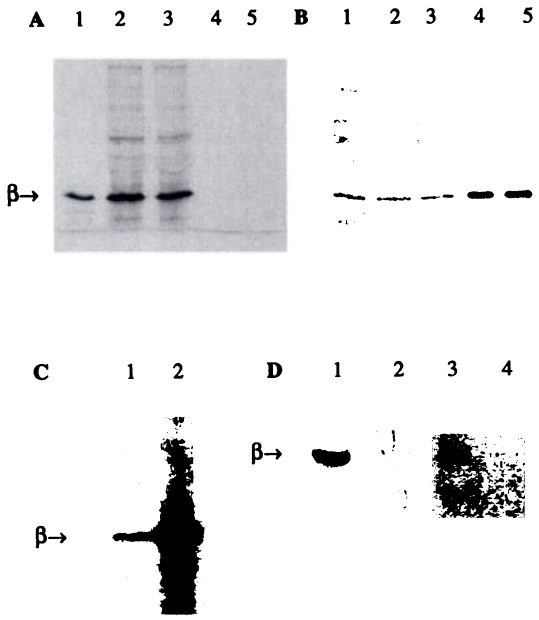

Fig. 1. Thiophosphorylation of the G protein β subunit in human platelet membranes in the presence of [^{35}S]GTPγS. A, Human platelet membranes (34 μg) were incubated in buffer containing 50 mM triethanolamin · HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM $MgCl_2$, and 0.1 μM [^{35}S]GTPγS (*lane 1*). *Lanes 2 and 3*, samples contained additional 50 mM $MgSO_4$ or the combination of 50 mM $MgSO_4$ and 10 μM epinephrine, respectively. *Lanes 4 and 5*, 2 μg of purified βγ subunits were incubated in the same buffer containing 50 mM $MgSO_4$ in the absence and presence of 1 μg $rG_{s\alpha\text{-}s}$, respectively. After 10 min at 22°, the samples were resolved on an 11% SDS polyacrylamide gel and transferred to nitrocellulose membranes. B, Immunoblot of the labeled proteins. Sequential incubation of the nitrocellulose membrane with the G protein β subunit-specific antiserum BN2 and an alkaline phosphatase-conjugated second antibody was used to visualize the immunoreactive bands. C, Immunoprecipitation of the [^{35}S]thiophosphorylated G protein β subunit. Platelet membranes (244 μg) were incubated with 100 nM [^{35}S]GTPγS for 10 min at 22° and subsequently solubilized in 250 μl of 10 mM Tris·HCl, pH 8, 25 mM EDTA, 0.2 mM PMSF, and 0.8% SDS. Immunoprecipitation was carried out as described under Experimental Procedures with 5 μl of antiserum 7. The immunoprecipitated material was applied to an 11% SDS-polyacrylamide gel (*lane 1*). *Lane 2*, starting material (50 μg membranes). D, Release of [^{35}S]thiophosphate by heat and inhibition of [^{35}S]thiophosphorylation of the G protein β subunit by pretreatment with DEPC. Platelet membranes (20 μg) were subjected to thiophosphorylation and subsequently incubated at 90° for 5 min (*lane 2*). *Lane 4*, before thiophosphorylation, the membranes were pretreated with 5 mM DEPC as outlined under Experimental Procedures; the corresponding control reactions are shown in *lanes 1 and 3*. The autoradiograms were obtained after a 16-hr (A and D) or 48-hr (C) exposure of the film (Kodak XAR 5) with an intensifying screen.

If purified βγ-dimers are incubated with [^{35}S]GTPγS, no thiophosphorylation of the β subunit is observed. Similarly, the addition of purified $rG_{s\alpha\text{-}s}$ to a reaction mixture containing [^{35}S]GTPγS and βγ-dimers under conditions in which the proteins are known to interact (25) does not lead to incorporation of thiophosphate into the β subunit (Fig. 1A, *lanes 4 and 5*). Thiophosphorylation of the G protein β subunit was also not observed if the free magnesium concentration varied between 0.1 and 9 mM and if myristoylated $rG_{i\alpha\text{-}1}$ and $rG_{i\alpha\text{-}2}$ were used as potential thiophosphate donors. Similarly, if G protein α subunits were allowed to bind [^{35}S]GTPγS to equilibrium and subsequently incubated with an excess of βγ-dimers, no labeling of the G protein β subunit was obtained (not shown). These findings rule out the ability of βγ-dimers to catalyze an autophosphorylation reaction and the ability of G protein α subunits to act as the source of thiophosphate and transfer it to the β subunit.

The radioactivity incorporated into the β subunit is easily released by heating (Fig. 1D, *lanes 1 and 2*) or acid treatment (not shown), suggesting that an amino acid residue other than serine, threonine, or tyrosine is phosphorylated. We therefore used DEPC to test whether the incorporation occurred on a histidine residue (26). A brief preincubation of human platelet membranes with DEPC, which results in the formation of *N*-carbethoxyhistidyl derivatives, completely abolished the subsequent incorporation of [^{35}S] into the G protein β subunit (Fig. 1D, *lanes 3 and 4*).

To evaluate the nucleotide specificity of the reaction, we compared the ability of unlabeled adenine and guanine nucleotides to block the incorporation of thiophosphate into the G_β band (Fig. 2). Inhibition of [^{35}S]thiophosphorylation is observed at 10-fold lower concentration of GTP than of ATP. Similarly, GDP was more potent than ADP in inhibiting thiophosphorylation (Fig. 2, *lanes 2 and 3*) and was more effective than an equimolar concentration of GTP (Fig. 2, *lanes 2 and 5*). This may reflect rapid and guanine nucleotide-dependent removal of the thiophosphate from the β subunit in the presence of nucleoside diphosphates. However, under the conditions used, one cannot distinguish between a competition of the nucleoside diphosphates for thiophosphorylation or an acceleration of the rate of dephosphorylation. To differentiate between these two possibilities, platelet membranes were incubated in the presence of [^{35}S]GTPγS, the free nucleotide was subsequently removed, and the nucleoside diphosphates were added (Fig. 3A). In the presence

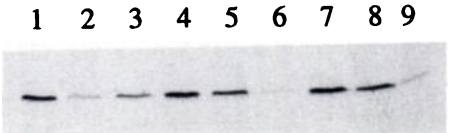

Fig. 2. Effect of nucleotides on the thiophosphorylation of G protein β subunits in platelet membranes. Platelet membranes (28 μg) were incubated with 0.1 μM [^{35}S]GTPγS for 15 min at 22° in the absence (*lane 1*) and presence of 1, 10, and 100 μM GTP (*lanes 4–6*); 0.01, 0.1, and 1 mM ATP (*lanes 7–9*); 10 μM GDP (*lane 2*); and 100 μM ADP (*lane 3*). The film was exposed for 16 hr.

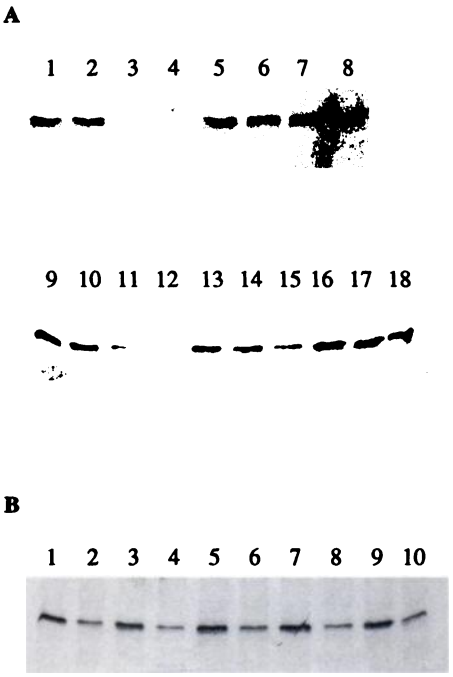

Fig. 3. A, Dephosphorylation of the [^{35}S]thiophosphorylated G protein β subunit in the presence of nucleoside diphosphates. The thiophosphorylation reaction was carried out for 5 min at 22° (*lane 1*). Subsequently, the free [^{35}S]GTPγS was removed by centrifugation, and the membranes were resuspended in buffer. Incubation was carried out for 5 min at 22° in the absence (*lanes 2 and 9*) and presence of nucleotides as follows. GDP: 1 μM (lane 10), 10 μM (*lanes 3 and 11*), and 100 μM (*lanes 4 and 12*); ADP: 1 μM (*lane 13*), 10 μM (*lanes 4 and 14*), and 100 μM (*lanes 6 and 15*); guanosine-5′-(2-*O*-thio)diphosphate: 10 μM (*lane 7*) and 100 μM (*lane 8*); and UDP: 100 μM (*lane 16*), 1 mM (*lane 17*), and 10 mM (*lane 18*). The reaction was stopped with Laemmli sample buffer, and the samples were applied to an 11% SDS-polyacrylamide gel. B, Effect of receptor agonists on thiophosphorylation and dephosphorylation of G protein β subunits in human platelet membranes. The conditions for incubation were as in A except that 1 μM GDP was present in the dephosphorylation reaction. *Lanes 1 and 2*, control thiophosphorylation and dephosphorylation. *Lanes 3, 5, 7, and 9*, thiophosphorylation in the presence of 10 μM NECA, 10 μM UK 14,304, 10 munits/ml thrombin, and 10 munits/ml insulin, respectively. *Lanes 4, 6, 8, and 10*, corresponding dephosphorylation reactions. The autoradiograms were obtained after a 16-hr exposure.

of 100 μM GDP, thiophosphorylated β subunit was almost completely dephosphorylated after 5 min, whereas ADP had only a modest effect, and guanosine-5′-(2-*O*-thio)diphosphate and UDP were ineffective. Agonists of G protein-coupled receptors have been shown to enhance the incorporation of [^{35}S] into the G protein β subunit in HL-60 membranes, and this effect has been suggested to result from an inhibition of the rate of dephosphorylation (27). We failed to find evidence of G protein-coupled receptor-dependent regulation of the rate of thiophosphorylation of the G protein β subunit in platelet membranes regardless of whether the rate of phosphorylation was determined in the absence or presence of GDP (Fig. 3B) for the A_2 adenosine receptor agonist NECA, the α_2-adrenergic agonist UK 14,304, or thrombin. Earlier reports have suggested that a protein related to the β subunit may be phosphorylated in response to prototypical receptor tyrosine kinases such as the epidermal growth factor receptor (28), and histidine phosphorylation of a 36-kDa membrane protein in response to epidermal growth factor has recently been reported in liver membranes (29). We therefore also tested insulin, the receptor of which is present on platelet membranes (30). However, insulin did not affect thiophosphorylation (Fig. 3B).

Phosphorylation on histidine residues is transient and usually followed by transfer to either a different amino acid, e.g., aspartate in the two-component system in bacteria (31, 32), or to nucleotides, e.g., during the reaction mechanism of NDP-kinase (33). The transfer of this thiophosphate to other amino acid residues should lead to the appearance of additional radioactively labeled proteins in the autoradiogram. However, no hint of such a mechanism was observed. Another possible explanation is thiophosphate transfer to nucleotides bound by nucleotide binding proteins. After gel electrophoresis under denaturating conditions, the nucleotides are released and a possible transfer reaction cannot be observed. To circumvent this limitation, an experimental protocol was chosen in which nucleotides are covalently incorporated into their corresponding proteins. Pretreatment of membranes with periodate results in the *in situ* oxidation of the 2′- and 3′-ribosyl hydroxyl groups of nucleotides. The aldehyde groups form Schiff's bases with the ϵ-amino group of lysine side chains, which on reduction with $NaCNBH_3$ and $NaBH_4$ are converted into covalent bonds (19, 23, 34). The addition of [^{35}S]GTPγS to platelet membranes that had first been subjected to sequential oxidation and reduction resulted in a dramatic change in the labeling pattern (Fig. 4A). Radioactivity was incorporated into many protein bands; in particular, a 45–47-kDa protein was the most prominent thiophosphate acceptor under these conditions, whereas labeling of the G protein β subunit was less intense. In native membranes, thiophosphorylation of the G protein β subunit occurred very fast, so that within 1 min, the maximum of [^{35}S]labeling was obtained. Labeling remained constant up to 45 min (Fig. 4B, *lanes 1–4*). In contrast, in sequentially oxidized and reduced membranes, incorporation of [^{35}S]thiophosphate into the additional proteins proceeded with slower rates, whereas that into the G protein β subunit gradually declined (Fig. 4B, *lanes 5 and 6*).

This thiophosphate transfer reaction observed in sequentially oxidized and reduced membranes may have resulted from denaturation of the proteins or from access of free reactive groups within the proteins induced by the agents used ($NaIO_4$, $NaCNBH_3$, and $NaBH_4$). The following control experiments were performed to rule out these nonspecific effects. (i) The order of addition of the reagents to the membranes was reversed, i.e., $NaBH_4$, followed by $NaCNBH_3$ and $NaIO_4$. Under these conditions, the labeling pattern is similar to that seen in native membranes, with the G protein β subunit being the predominantly labeled protein (Fig. 4C, *lanes 5 and 6*). Identical results were obtained when membranes were treated with a mock solution consisting of premixed $NaIO_4$, $NaCNBH_3$, and $NaBH_4$ (data not shown). (ii) Sequentially oxidized and reduced platelet membranes were preincubated with the 2′,3′-dialdehyde GTPγS analogue oGTPγS, which is a quasi-irreversible ligand of G proteins (19). Pretreatment with oGTPγS completely blocked the subsequent [^{35}S]thiophosphate incorporation into the additional protein bands. This inhibition was observed regardless of whether the membranes were preincubated with oGTPγS (Fig. 4C, *lane 3*) or the pretreatment was followed by subsequent oxidation and reduction (Fig. 4C, *lane 2*). (iii) If [^{35}S]GTPγS was added first and the membranes subse-

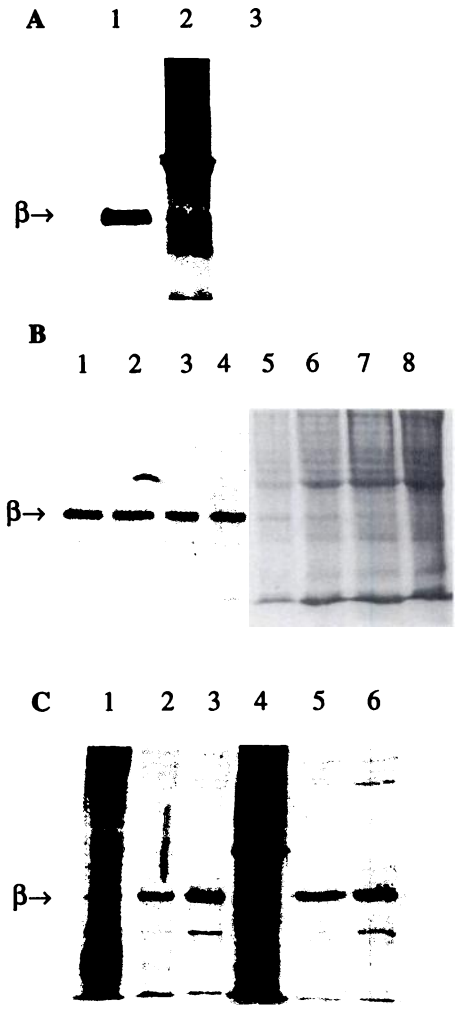

Fig. 4. [^{35}S]Thiophosphate labeling in membranes subjected to sequential oxidation and reduction. A, *lane 1*, thiophosphorylated membranes (control reaction). *Lane 2*, thiophosphorylation in oxidized and reduced membranes. Native human platelet membranes (*lane 1*) or membranes subjected to sequential oxidation with $NaIO_4$ and reduction with $NaCNBH_3$ and $NaBH_4$ (*lanes 2 and 3*) were preincubated in the absence (*lanes 1 and 2*) or presence (*lane 3*) of 5 mM DEPC. After removal of DEPC, membranes (40 μg) were incubated in the presence of 0.1 μM [^{35}S]GTPγS for 5 min at 22°. The samples were dissolved in Laemmli sample buffer and resolved on an 11% SDS-polyacrylamide gel. The autoradiograms were obtained after a 16-hr exposure. B, Time course of [^{35}S]thiophosphate incorporation in native human platelet membranes and membranes subjected to sequential oxidation and reduction. Native membranes (*lanes 1–4*) and oxidized and reduced membranes (*lanes 5–8*) were incubated with 0.1 μM [^{35}S]GTPγS. At 1 (*lanes 1 and 5*), 5 (*lanes 2 and 5*), 15 (*lanes 3 and 7*), and 45 min (*lanes 4 and 8*), aliquots of the reaction mixture (40 μg protein) were withdrawn and subjected to SDS-gel electrophoresis. The autoradiogram was obtained after an exposure time of 48 hr. C, Specificity of the [^{35}S]thiophosphate labeling in sequentially oxidized and reduced human platelet membranes. Native platelet membranes (*lane 6*) or platelet membranes (20 μg) subjected to sequential oxidation with $NaIO_4$ and reduction with $NaCNBH_3$ and $NaBH_4$ (*lane 4*) were incubated in the presence of 0.1 μM [^{35}S]GTPγS. *Lanes 2 and 3*, membranes were preincubated with 100 μM oGTPγS for 5 min at 22°. Thereafter, unbound oGTPγS was removed by centrifugation; membranes were either immediately incubated in the presence of 100 nM [^{35}S]GTPγS (*lane 3*) or first oxidized with $NaIO_4$ and reduced with $NaCNBH_3$ and $NaBH_4$, followed by centrifugation and the addition of 0.1 μM [^{35}S]GTPγS (*lane 2*). *Lane 5*, the order of addition was $NaBH_4$, followed by $NaCNBH_3$ and $NaIO_4$. *Lane 1*, native membranes were first incubated in the presence of 100 nM [^{35}S]GTPγS and subsequently oxidized, reduced, and resuspended. The autoradiogram was obtained after a 16-hr exposure.

quently oxidized and reduced, a different labeling pattern was observed (Fig. 4C, *lane 1*). Under these conditions, not only thiophosphorylated proteins but also proteins that bind [^{35}S]GTPγS are visualized. This is exemplified by the lack of radioactive labeling of the 45-kDa protein and the many other proteins that were seen in the sample that had first been sequentially oxidized and reduced (Fig. 4C, compare *lanes 1 and 4*) and by the predominant incorporation of radioactivity into proteins migrating in the 20–30-kDa region. These proteins most likely correspond to the low molecular mass *ras*-like G proteins, which are abundant in platelets. Labeled proteins are also detected in the 100-kDa region. Furthermore, [^{35}S]oGTPγS-labeled G proteins can be extracted from these membranes and immunoprecipitated with specific antisera (see below). (iv) Exposure of platelet membranes to DEPC before sequential oxidation and reduction completely abolished the subsequent thiophosphorylation of the G protein β subunit as well as the [^{35}S]labeling of the other proteins (Fig. 4A, *lane 3*). (v) In addition, incorporation of [^{35}S]thiophosphate into the proteins of native or sequentially oxidized and reduced membranes was not affected by the addition of calcium, a combination of calcium and calmodulin, phorbol esters, the specific peptide inhibitors for protein kinase C, protein kinase A, and calmodulin-dependent kinase II. Similarly, tyrosine kinase inhibitors, such as genistein and tyrphostin, and staurosporin also failed to produce an effect on both reactions (data not shown).

Taken together, these data indicate that in oxidized and reduced membranes, a specific thiophosphate incorporation can be observed that presumably requires nucleotides prebound to acceptor proteins and thus may reflect a thiophosphate transfer reaction. The results also imply that a histidine residue, most likely on the G protein β subunit, is required for this transfer reaction. To verify that the transphosphorylation reaction is mediated by the G protein β subunit, platelet membranes were [^{35}S]thiophosphorylated and the free nucleotide was removed by centrifugation and washing, resulting in an overall dilution to <1 nM free [^{35}S]GTPγS. Solubilization of these [^{35}S]thiophosphate donor membranes with 12.5 mM CHAPS results in the almost-complete recovery of the [^{35}S]thiophosphorylated β subunit in the soluble supernatant (Fig. 5, *lanes 1 and 2*). If this soluble supernatant, which contained the [^{35}S]thiophosphorylated β subunit as the predominant source of radioactivity (Fig. 5, *lane 4*), was added to sequentially oxidized and reduced membranes, a reduction in the [^{35}S]thiophosphate in the β subunit was accompanied by an incorporation into other proteins, mainly, a 45-kDa protein and a doublet protein band in the 100-kDa mass range (Fig. 5, *lane 5*). Although this transfer reaction resulted in rather weak labeling, requiring prolonged exposure, the pattern was similar to that observed in the control reaction (Fig. 5, *lane 8*, but compare with Fig. 4A, *lane 2*).

Sequential oxidation and reduction essentially abolished adenylyl cyclase activity in human platelet membranes, so it was not possible to assess the functional effect of a phosphate transfer reaction for G protein-mediated regulation of the enzyme in oxidized and reduced membranes. We therefore briefly preincubated platelet membranes with oGTP and compared the abilities of GTPγS and Gpp(NH)p to inhibit adenylyl cyclase. The presence of an ATP-regenerating system has been shown to mask the contribution of transphosphorylation to adenylyl cy-

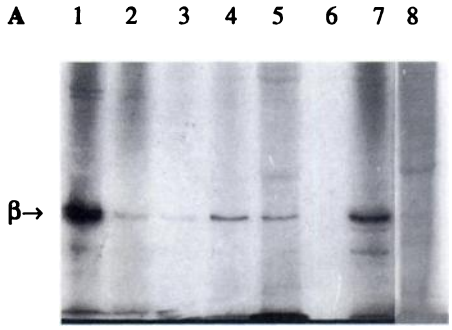

Fig. 5. Transfer of [^{35}S]thiophosphate by a soluble preparation containing [^{35}S]thiophosphate-labeled G protein β subunit to sequentially oxidized and reduced human platelet membranes. Then, 400 μg of native platelet membranes was labeled with 100 nM [^{35}S]GTPγS. The unbound [^{35}S]GTPγS was removed by centrifugation and washing. The membranes were subsequently solubilized by homogenization in 0.4 ml TEMN containing 12.5 mM CHAPS. The insoluble material and the solubilized material were separated by centrifugation, and 10% aliquots of insoluble and solubilized material were applied in *lanes 1 and 2*, respectively. *Lane 3*, a corresponding aliquot of CHAPS-insoluble material was thiophosphorylated after the membranes had been solubilized. The solubilized material shown in *lane 2* was concentrated 10-fold by ultrafiltration over a Centricon 30, and a 10% aliquot was applied in *lane 4*. A 10% aliquot of this concentrate was combined with 20 μg of sequentially oxidized and reduced membranes (*lane 5*). *Lane 6*, a 10% aliquot of the ultrafiltrate from the solubilized supernatant was combined with sequentially oxidized and reduced platelet membranes to control for the carryover of residual free [^{35}S]GTPγS. *Lanes 7 and 8*, control reaction in native and sequentially oxidized and reduced platelet membranes (20 μg) in the presence of 100 nM [^{35}S]GTPγS (*lane 8* was deliberately underexposed during photographic reproduction). The samples were resolved on an 11% SDS-polyacrylamide gel. The autoradiogram was obtained after a 4-day exposure.

clase inhibition (2). Therefore, very low amounts of platelet membrane protein were used, and the ATP-regenerating system was omitted. Because the imido-bond between β- and γ-phosphate in Gpp(NH)p is highly resistant to cleavage by hydrolytic enzymes, differences between GTPγS and Gpp(NH)p suggest participation of phosphate transfer reactions in the G protein-dependent inhibition of adenylyl cyclase (2). In the absence of the quasi-irreversible G protein ligand oGTP, both GTPγS and Gpp(NH)p inhibited adenylyl cyclase activity. The concentration of GTPγS required for half-maximum inhibition of adenylyl cyclase was 13 ± 3 nM in the absence and 3 ± 1 nM in the presence of the α_2-adrenoceptor agonist UK 14,304. If membranes were briefly preincubated with oGTP, GTPγS still supported adenylyl cyclase inhibition, whereas Gpp(NH)p had no effect (Fig. 6A). In addition, the concentration-response curve of GTPγS (Fig. 6B) was shifted to the left if the platelet α_2-adrenergic receptor was activated by the agonist UK 14,304 (Fig. 6B). Both the inhibitory effect of GTPγS and the receptor-dependent increase in potency were blocked by 1 mM UDP (Fig. 6B). The interpretation of the experiment is complicated by the progressive exchange of prebound GDP for quasi-irreversibly binding oGTP during the time course of the reaction. Nevertheless, our observations are indicative of the receptor-stimulated phosphate transfer reaction as described previously. This reaction can be observed in the presence of the oxidized GTP analogue oGTP.

Because direct transfer of (thio)phosphate from the G protein β subunit to G protein α subunits has been postulated (6, 27, 35), we searched for possible labeling of $G_{s\alpha}$ and $G_{i\alpha\text{-}2}$, which are the candidate acceptors in human platelet membranes (22). We tried to immunoprecipitate $G_{s\alpha}$ and $G_{i\alpha\text{-}2}$ with specific antisera from sequentially oxidized, reduced, and [^{35}S]thiophosphorylated platelet membranes. The immunoprecipitation did not result in the recovery of any radioactively labeled proteins that comigrated with [^{35}S]oGTPγS-labeled purified $rG_{s\alpha\text{-}L}$ and $rG_{i\alpha\text{-}2}$ (Fig. 7A), although large amounts of membrane extracts were used as starting material. The presence of $G_{s\alpha\text{-}s}$, $G_{s\alpha\text{-}L}$, and $G_{i\alpha}$ in the immunoprecipitate was separately verified by immunoblotting of the immunoprecipitate (not shown). Most important, the antisera used immunoprecipitated [^{35}S]oGTPγS-labeled $G_{s\alpha\text{-}s}$, $G_{s\alpha\text{-}L}$, and $G_{i\alpha\text{-}2}$ from membranes in which prebound GDP was first exchanged for [^{35}S]GTPγS and then covalently incorporated by oxidation of the ribose ring and reduction of the Schiff's base (Fig. 7B).

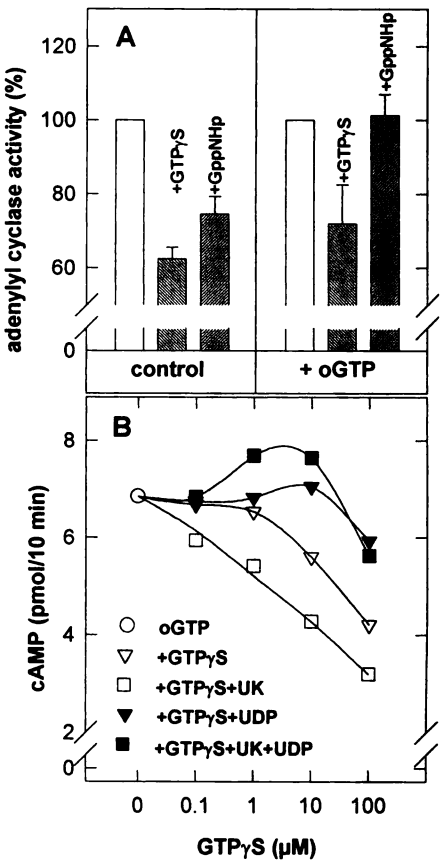

Fig. 6. Inhibition of forskolin-stimulated adenylyl cyclase activity in the absence and presence of oGTP. A, Platelet membranes (5–7 μg) were preincubated in the absence (*control*) and presence (*+oGTP*) of 10 μM oGTP for 5 min, followed by the addition of 10 μM GTPγS or of 10 μM Gpp(NH)p as outlined in Experimental Procedures. Activity stimulated by forskolin (50 μM) in the absence and presence of oGTP was 411 ± 60 and 165 ± 46 pmol/min/mg, respectively, and was set 100% (*open bars*) to normalize for variations between individual preparations. Data are given as mean ± standard deviation from four experiments done in duplicate. B, Platelet membranes (5 μg) were preincubated with 10 μM oGTP, followed by the addition of GTPγS (▽), GTPγS + 10 μM UK 14,304 (□), GTPγS + 1 mM UDP (▼), or GTPγS + 10 μM UK 14,304 + 1 mM UDP (■). Data are mean values from duplicate determinations in a single experiment that was repeated twice.

Discussion

Thiophosphorylation of the G protein β subunit has previously been reported for transducin $\beta\gamma$-dimer and for the G

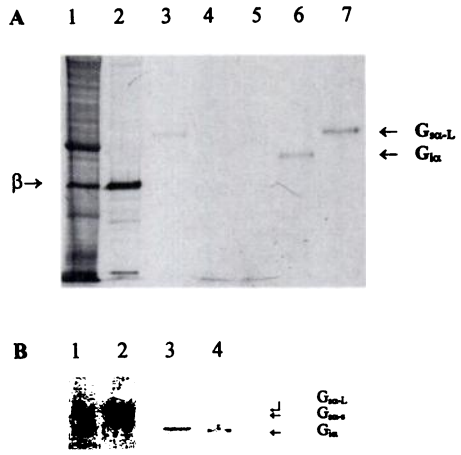

Fig. 7. Immunoprecipitation of G protein α subunits from [^{35}S]thiophosphate-labeled human platelet membranes subjected to sequential oxidation and reduction (A) and after [^{35}S]oGTPγS labeling (B). A, Sequentially oxidized and reduced platelet membranes (400 μg) were incubated with 0.1 μM [^{35}S]GTPγS and solubilized as described in Experimental Procedures. After preclearing with protein A-conjugated Sepharose beads, the solubilized supernatant was incubated for 2 hr at 4° with antisera 333 (specific for $G_{s\alpha}$, *lane 5*) and AS7 (specific for $G_{i\alpha\text{-}2}$/$G_{i\alpha\text{-}1}$, *lane 4*) (10 μl each); protein A-conjugated Sepharose beads (~4 μg) were allowed to bind the antibodies for 1 hr at 4°. Proteins were resolved on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose. *Lanes 2 and 1*, control reaction in native platelet membranes and oxidized and reduced membranes, respectively. *Lanes 3 and 7*, 20 and 100 ng [^{35}S]oGTPγS-labeled $rG_{s\alpha\text{-}L}$, respectively. *Lane 6*, 100 ng [^{35}S]oGTPγS-labeled $rG_{i\alpha\text{-}2}$. B, Platelet membranes (100 μg) were incubated with 0.1 μM [^{35}S]GTPγS for 10 min at 20°. After *in situ* oxidation, membranes were solubilized, and immunoprecipitation carried out as in A with antisera 333 (*lane 1*) and AS7 (*lane 3*) (2.5 μl each). *Lane 2*, a combination of o[^{35}S]GTPγS-labeled $rG_{s\alpha\text{-}s}$ (10 ng) and $rG_{s\alpha\text{-}L}$ (100 ng). *Lane 4*, o[^{35}S]GTPγS-labeled $rG_{i\alpha\text{-}1}$ (100 ng). The autoradiograms were obtained after a 48-hr exposure.

protein β subunits expressed in the human leukemia cell line HL60 (6, 35). When added to human platelet membranes, purified thiophosphorylated transducin βγ is capable of mimicking the action of GTPγS in the bidirectional regulation of adenylyl cyclase, i.e., stimulation of basal and inhibition of forskolin-stimulated enzyme activity (6). The model, which has been proposed to account for all experimental observations, assumes that after receptor-dependent stimulation of GDP release and binding of GTP or GTPγS, G protein α subunits can transfer the terminal phosphate or thiophosphate to the β subunit. This (thio)phosphate is subsequently directly transferred to the next α subunit, and this reaction was presumed to contribute to amplification of the receptor-generated signal (27).

We characterized the GTPγS-dependent thiophosphorylation in human platelet membranes and developed an experimental strategy to search directly for thiophosphate transfer reactions to protein-bound nucleotides. As in HL60 membranes, the G protein β subunit was identified as the major substrate for GTPγS-dependent thiophosphorylation. The conclusion is supported by the comigration of the thiophosphorylated protein with purified β subunits, immunoprecipitation, and immunoblotting with two different antisera of the thiophosphorylated protein. The thiophosphorylation reaction involves a histidine residue and is specific for guanine nucleotides. However, the apparent selectivity is much lower than that reported in HL60 cell membranes, where thiophosphorylation of the G protein β subunit has been described as highly specific for guanine nucleotides (35). A possible explanation may be a high rate of conversion of ATP to GTP catalyzed by a NDP-kinase, which is abundant in platelet membranes (data not shown, see Ref. 36). In contrast to HL60 membranes, no effect of receptor agonists, such as activation of A_2 adenosine receptor (G_s-coupled), α_2-adrenergic receptor (G_i-coupled), and thrombin receptor (G_i-, G_q-, and $G_{12/13}$-coupled) was detected in platelet membranes. The extent of thiophosphorylation or dephosphorylation was not altered by any of the investigated G protein-coupled receptor agonists. Thus, the basic features of β subunit thiophosphorylation, i.e., modification of a histidine residue and guanine nucleotide specificity of the phosphorylation and dephosphorylation reaction, are similar in HL60 membranes and in human platelet membranes, but differences remain, which we currently cannot explain.

Nevertheless, our results clearly refute the postulated model of a direct phosphate transfer from the G protein β subunit to GDP liganded to the nucleotide binding pocket of G protein α subunits. We tested three aspects that are central to this transphosphorylation hypothesis: (i) the presence of a phosphotransfer reaction which is functionally relevant to effector regulation, resulting from (ii) the α subunit-catalyzed (thio)phosphorylation of the β subunit and (iii) the subsequent direct transfer from the β subunit to GDP bound to α subunits. We verified that GTPγS supported inhibition of adenylyl cyclase in a receptor-regulated manner in oGTP-pretreated membranes. The effect of GTPγS was blocked by UDP, and inhibition was not mimicked by Gpp(NH)p. These findings confirm that the phosphotransfer reaction, which has been characterized in platelet membranes (2), is detectable in the presence of the oxidized GTP analogue oGTP. However, our observations show that this phosphotransfer reaction is unrelated to the thiophosphorylation of the β subunit. This conclusion is based on the findings that neither thiophosphorylation of the β subunit is either stimulated by receptor agonists, including UK 14,304, nor is this reaction blocked by UDP. In addition, if membranes are pretreated with oGTP for prolonged incubation times to fully inactivate G_i and free oGTP is removed, subsequent addition of GTPγS neither inhibits nor stimulates adenylyl cyclase (see Ref. 22). This rules out the (thio)phosphate transfer reaction occurring directly on GDP bound to the G protein α subunit and argues for a thiophosphate transfer reaction to form oGTPγS in solution. Similarly, the second assumption of the direct phosphate transfer hypothesis can be ruled out; incubation of purified βγ-dimers with purified G protein α subunits, in particular, $rG_{s\alpha\text{-}s}$, in which the spontaneous exchange of nucleotides occurs rapidly (17), did not lead to any thiophosphorylation of the β subunit. The experimental strategy used to verify the third aspect of the transphosphorylation model involves covalent incorporation of bound nucleotides in potential acceptor proteins (19, 23, 34). Thus, potential artifacts are avoided that may result from the continuous release of protein-bound GDP and its subsequent (thio)phosphorylation by NDP-kinase (13–16). Under conditions, where the GDP-release reaction cannot interfere, multiple proteins are radioactively labeled in platelet membranes. Nevertheless, the thiophosphate was not recovered on either $G_{s\alpha}$ or $G_{i\alpha}$; these should be the candidate acceptor G protein α subunits if the ability of thiophosphorylated transducin βγ-dimers to stimulate and inhibit platelet adenylyl cyclase is explained by the

direct phosphate transfer reaction (6). Therefore, we conclude that G protein α subunits in platelet membranes are not the acceptor of the β subunit-dependent thiophosphate transfer reaction and that G protein activation requires GDP release and rebinding of a guanine nucleotide triphosphate formed in solution.

The fate of the (thio)phosphate on the G protein β subunit, and thus the downstream-signaling properties, is not clear. Although detergent extracts containing the labeled G protein β subunit act as a source of thiophosphate for transfer reactions, this does not rule out that proteins other than the G protein β subunit actually catalyze this reaction. Reconstitution of the transfer reaction with purified components, i.e., the enzyme(s) catalyzing the guanine nucleotide-specific (thio)phosphorylation of G_β and acceptor protein(s), are required to firmly establish the role of $\beta\gamma$-dimers in the phosphate transfer reaction. We believe that the experimental strategy outlined in the present study represents a basis for the identification of new proteins that are potential targets for regulation by $\beta\gamma$-dimers.

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