Species- and tissue-dependent diversity of G-protein β subunit phosphorylation: evidence for a cofactor

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We previously reported that, in the membranes of HL-60 cells during activation of G-proteins, a phosphate transfer reaction occurs which involves transient G-protein β subunit (Gβ) phosphorylation [Wieland, Nürnberg, Ulibarri, Kaldenberg-Stasch, Schultz and Jakobs (1993) J. Biol. Chem. **268**, 18111–18118]. Here, the generality of this phenomenon is evaluated by studying membranes of various tissues obtained from different mammalian species. All membranes tested expressed at least Gβ_1 and Gβ_2 subunits. Cell membranes from bovine and porcine brain and liver, rat brain and human blood cells exhibited predominantly Gβ_1 or both subtypes at roughly equal concentrations. In contrast, significantly more Gβ_2 immunoreactivity was detected in membranes from human placenta. Bovine and porcine liver membranes exhibited weak Gβ-specific immunoreactive signals. Conversely, these membranes showed the highest levels of Gβ phosphorylation after incubation with [γ-^{32}P]GTP or ^{35}S-labelled guanosine 5′-[γ-thio]triphosphate. Interestingly, Gβ-specific phosphorylation of membranes from human erythrocytes and platelets was very weak. Gβ phosphorylation was confirmed by immunoprecipitation with Gβ-specific antibodies, and the target amino acid was identified as histidine. On SDS/PAGE, phosphorylated or thiophosphorylated Gβ-proteins differed in their apparent molecular size from unmodified Gβ-proteins. Moreover, phosphorylated Gβ-proteins differed in a species-dependent fashion in their electrophoretic mobility. Solubilization of membrane proteins with detergent did not abolish Gβ phosphorylation. In contrast, reconstituted purified G_i/G_o proteins showed no Gβ phosphorylation. From these experiments we conclude that: (i) Gβ phosphorylation represents a general phenomenon occurring in the cells of various species to different degrees, (ii) phosphorylated Gβ-proteins exhibit species-dependent diverse electrophoretic mobilities, and (iii) Gβ phosphorylation requires a membrane-associated cofactor(s) which is lost during routine G-protein purification.

INTRODUCTION

G-proteins are molecular switches that transfer extracellular signals from a plethora of ligand-activated heptahelical membrane receptors to an array of effector systems including enzymes and ion channels (for recent reviews see [1,2]). They are composed of α, β and γ subunits and are classified by the nature of their α subunit. So far, cDNAs of 23 distinct Gα, at least five Gβ and 11 Gγ subunits have been identified. To function as reversible signal transducers, G-proteins undergo a cycle of active and inactive states [3]. In its inactive form, Gα binds GDP and is associated with the G$\beta\gamma$ dimer. Activation of membrane receptors results in an exchange of GDP for GTP and dissociation of G$\beta\gamma$ from Gα. This allows the interaction of Gα and/or G$\beta\gamma$ with effectors. Eventually, signalling is terminated by the intrinsic GTPase activity located on Gα, followed by reassociation of the GDP-bound Gα with the G$\beta\gamma$ dimer.

In addition to this well established mechanism for the activation of G-proteins, we have observed an alternative route of activation in HL-60 cell membranes [4–7] in which Gβ subunits function as high-energy-phosphate acceptors. Phosphorylation of Gβ presumably occurs at a histidine residue, allowing rapid phosphate transfer to a second GDP, possibly bound to Gα. In fact, thiophosphorylated transducin Gβ was able to form guanosine 5′-[γ-thio]triphosphate (GTP[S]) from membrane-bound GDP in HL-60 and platelet membranes. This mechanism should, therefore, convey an alternative route of activation of G-proteins that avoids the GDP/GTP exchange step. Little is currently known about the generality of this mechanism.

Here we describe the phosphorylation of Gβ in different human cells and tissues and in various other mammalian tissues. Most tissues and cells tested showed significant Gβ phosphorylation. In addition, we provide evidence for the presence of an as yet unidentified cofactor that is necessary for Gβ phosphorylation.

EXPERIMENTAL

Materials

[^{35}S]GTP[S] (1150–1400 Ci/mmol) and [^{32}P]P_i (approx. 9000 Ci/mmol) were purchased from DuPont-New England Nuclear (Bad Homburg, Germany). [γ-^{32}P]GTP was synthesized as described [8], and each preparation was used for less than 3 weeks. Phosphoamino acid standards were from Sigma. Other reagents were from sources described previously [7,9,10], or were of the highest analytical grade quality.

Membrane preparations and G-protein purification

Outdated human erythrocytes and thrombocytes were obtained from a local blood bank, and human placentae were collected shortly after parturition. In addition, human platelet membranes were donated by Dr. U. Walter, University of Würzburg, Germany. Bovine and porcine tissues were supplied by a local slaughterhouse, and rat brains were obtained immediately after decapitation. HL-60 cells were grown in suspension culture and induced to differentiate into mature myeloid forms in the presence of 1.25 % (v/v) DMSO [6]. Cell membranes from various sources were prepared as described previously [9]. All membranes were

Abbreviations used: Gα, Gβ and Gγ, G-protein α, β and γ subunits respectively; GTP[S], guanosine 5′-[γ-thio]triphosphate.

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frozen in liquid nitrogen and stored at −80 °C until use. G_i/G_o-proteins were purified and reconstituted as detailed elsewhere [11,12].

SDS/PAGE, antibodies and immunoblotting

For detection of Gβ subunits, SDS/PAGE was employed. Separating gels contained 10 % (w/v) acrylamide, or 9 % (w/v) acrylamide when supplemented with 6 M urea. Immunoblotting was carried out as described [11], and filter-bound antibodies were visualized by a colour reaction catalysed by alkaline phosphatase or goat anti-(rabbit IgG) coupled to peroxidase (dilution 1:1000; Sigma, Deisenhofen, Germany) and the enhanced chemiluminescence (ECL) Western-blotting detection system (Amersham, Braunschweig, Germany). ECL-stained blots were exposed to X-ray films for 1–40 min. Generation of antisera AS11 (anti-G$\beta_{1/2/3/4}$), AS28 (anti-Gβ_1) and AS36 (anti-Gβ_2) was as previously described [13].

Protein phosphorylation and analysis by SDS/PAGE

Membrane proteins were phosphorylated or thiophosphorylated by incubation with 20 nM [γ-^{32}P]GTP or [^{35}S]GTP[S]. The reaction was started by addition of the membranes (approx. 20 μg of protein per tube) to the prewarmed reaction mixture and conducted for the indicated periods of time at 30 °C. The reaction was terminated by addition of an equal volume of 2 × concentrated SDS/PAGE sample buffer [14]. Subsequently, the samples were kept at room temperature for at least 1 h before loading on to polyacrylamide gels. In some experiments, proteins were transferred to nitrocellulose sheets by Western blotting. For analysis, filters or dried gels were exposed to X-ray films. Radioactive bands of interest were cut out and subjected to liquid scintillation spectrometry.

GTP[S] binding, protein thiophosphorylation and analysis by filtration

Membrane suspensions (40–80 μg) were incubated with a buffer containing [^{35}S]GTP[S] (500 nM; 100000 c.p.m.) in a final volume of 60 μl at 32 °C for 60 min, as described [11]. The reaction was terminated either by filtration through nitrocellulose filters for quantification of GTP[S] binding activity or by addition of an equal volume of 2 × concentrated SDS/PAGE sample buffer [14] for measurement of protein thiophosphorylation. In the latter case, samples were kept for another 15 min at room temperature before filtration through nitrocellulose filters. Washed filters were subjected to liquid scintillation spectrometry. Non-specific labelling was registered after co-incubation with 2 mM unlabelled GTP[S].

Immunoprecipitation of Gβ

Phosphorylation of membranes (200 μg of protein per tube) was induced by addition of [γ-^{32}P]GTP (100 nM) for 1 min at 30 °C. The reaction was stopped by placing the mixture on ice and adding an equal volume of 50 mM EDTA, pH 7.4. After centrifugation (13000 ***g***), membranes were solubilized at 4 °C using 40 μl of a mixture of 25 mM EDTA, pH 7.4, 0.8 % (w/v) SDS and 0.2 μM PMSF (final concentrations). Precipitation buffer (280 μl) {150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 25 mM EDTA, 1 mM dithiothreitol, 1 mM NaF, 1 % (w/v) sodium deoxycholate, 0.3 μM aprotinin, 0.2 μM PMSF, 10 μM GTP[S], 1 % (v/v) Nonidet P-40} was added to the solubilized membranes at 4 °C, followed by addition of 10 μl of the same buffer containing 1.25 mg of Protein A–Sepharose beads. After incubation and centrifugation (13000 ***g***, 5 min), the clear supernatant was taken and supplemented with 30 μl of rabbit antiserum and 50 μl of 10 % (w/v) Protein A–Sepharose beads. The mixture was gently shaken for 2.5 h at 4 °C. Protein A–Sepharose beads were pelleted and washed twice with precipitation buffer and twice with precipitation buffer containing 300 mM NaCl. Bound proteins were eluted by adding sample buffer [14] and loaded on to SDS/polyacrylamide gels.

Synthesis and analysis of phosphorylated amino acids

For preparation of the phosphorylated forms of the amino acids histidine and lysine, we followed a published protocol [15]. Modified amino acids were stored at −20 °C until use. Washed HL-60 membranes (2.3 mg of protein) were phosphorylated for 3 min and processed by SDS/PAGE and Western blotting as described above. The ^{32}P-phosphorylated G-protein band was excised, divided into 12 equal parts and incubated with 3 M KOH for 5 h at 105 °C. After neutralizing with HCl, the samples were subjected to TLC and subsequent analysis by the method of Motojima and Goto [16]. For identification of the phosphorylated amino acids, the mobilities of the radiolabelled samples were compared with those of phosphoprotein standards visualized by ninhydrin.

Miscellaneous

Protein concentrations were determined by the method of Lowry et al. [17], with modifications [18]. All experiments were repeated at least twice.

RESULTS

Immunodetection of Gβ in mammalian cell membranes

First, we analysed Gβ-immunoreactive proteins in membranes of various cells and tissues by employing specific antisera. Immunoblotting of membrane proteins exhibited two Gβ-immunoreactive bands after staining with AS 11 (anti-$\beta_{1/2/3/4}$ antibody; Figure 1, left lane of each group of three lanes). Of the two immunoreactive bands, only the upper one was detected by a Gβ_1-specific antibody (AS 28; see Figure 1, middle lane), whereas the lower band was sensitive to an antibody specific for Gβ_2 (AS 36; see Figure 1, right lane). It should be noted that addition of urea to SDS/PAGE resulted in a shift of mobility of Gβ

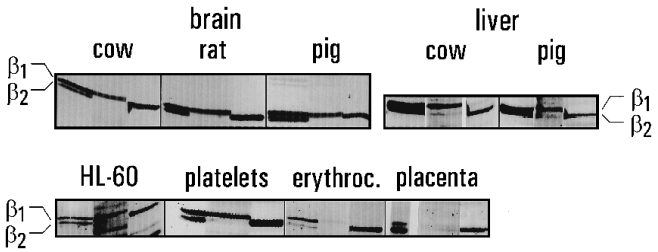

Figure 1 Immunodetection of Gβ

Acetone-precipitated animal and human (HL-60 cells, platelets, erythrocytes and placenta) membrane proteins (1 mg; 4 cm slots) were resolved on SDS/polyacrylamide gels containing 6 M urea and 9% acrylamide. After blotting, nitrocellulose filters were cut into strips and each set was incubated with AS11 (anti-G$\beta_{1/2/3/4}$ serum, diluted 1:250; left lane of each group of three), AS28 (anti-Gβ_1 serum, diluted 1:150; middle lane) or AS36 (anti-Gβ_2 serum, diluted 1:400; right lane). The ECL system was used to detect filter-bound antibodies. Nitrocellulose strips were exposed to X-ray films for 5 min, except those containing membrane proteins from bovine and porcine liver and human placenta which were exposed for 30 min.

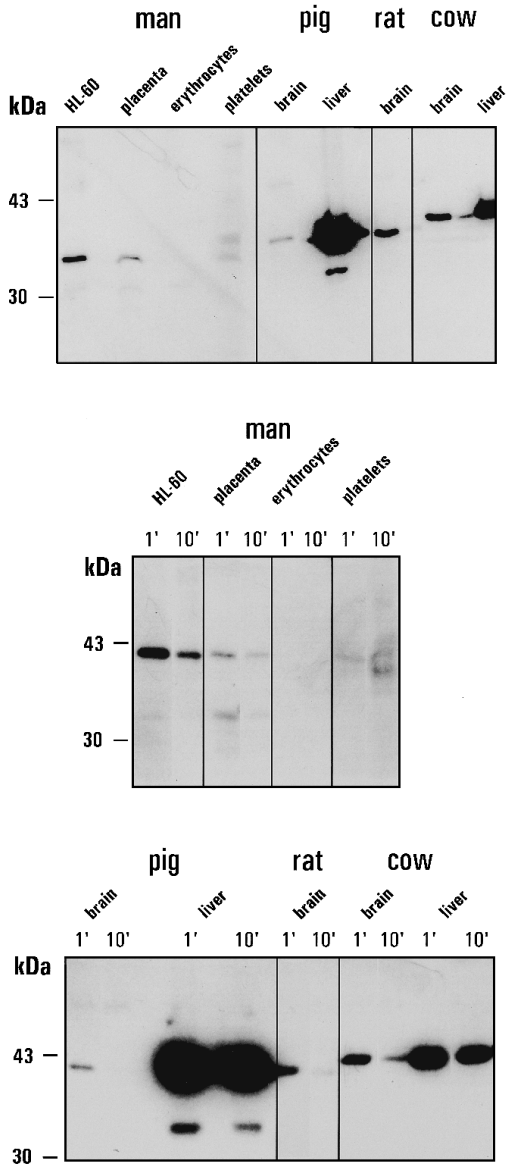

Figure 2 Phosphorylation of membrane-bound proteins

Membrane proteins (20 μg/tube) of different origin were phosphorylated with [γ-^{32}P]GTP (20 nM) as described in the Experimental section. Proteins were resolved on an SDS gel containing 10% acrylamide (top panel) or on an SDS gel containing 6 M urea and 9% acrylamide after phosphorylation for 1 min (1′) or 10 min (10′) (middle and bottom panels). Shown are the autoradiograms of the exposed gels. The molecular masses (kDa) of marker proteins are indicated.

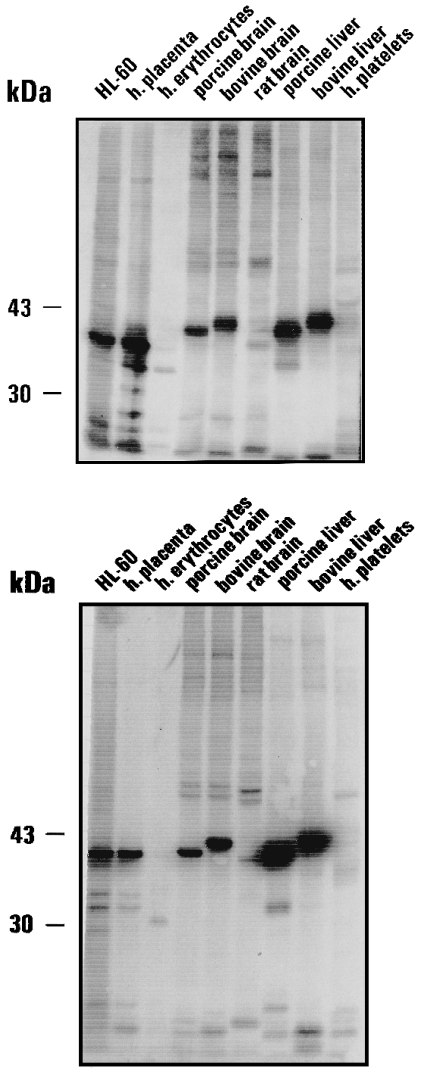

Figure 3 Thiophosphorylation of membrane-bound proteins

Membrane proteins (20 μg/tube) of different origin (h., human) were thiophosphorylated with [^{35}S]GTP[S] (20 nM) for 60 min as described in the Experimental section. Proteins were resolved on an SDS gel containing 10% acrylamide (upper panel) or on an SDS gel containing 6 M urea and 9% acrylamide (lower panel). Shown are the autoradiograms of the exposed gels. The molecular masses (kDa) of marker proteins are indicated.

proteins from an apparent molecular size of 35/36 kDa to 40/42 kDa, which allowed better resolution between $G\beta_1$ and $G\beta_2$ [10]. All membranes tested exhibited $G\beta_1$ and $G\beta_2$ immunoreactivity. The individual expression of the Gβ-proteins varied among the different sources (Figure 1). Clearly, membranes from bovine, porcine and rat brains showed strong immunoreactive signals. Moreover, in these tissues $G\beta_1$ immunoreactivity was equal to or exceeded $G\beta_2$ immunoreactivity as detected by antiserum AS11 which recognizes both subtypes. With membranes from bovine and porcine liver, only weak immunoreactive signals were visible after prolonged exposure to X-ray films. Qualitatively, the same expression pattern was found in almost all other tissues and cells tested. In contrast, placenta membranes expressed low concentrations of Gβ-immunoreactive proteins, of which $G\beta_2$ was the predominant one (Figure 1). In summary, these results confirmed previous reports from various laboratories [13,19].

Phosphorylation of membrane proteins

To evaluate the possible phosphorylation of Gβ [6], we phosphorylated membranes using [γ-^{32}P]GTP and performed size fractionation of proteins by SDS/PAGE. As demonstrated in Figure 2 (top panel), four observations were striking. (i) Most membranes exhibited only one radioactively labelled protein band, with apparent molecular masses ranging from 35 to 42 kDa. (ii) Phosphorylation of these proteins was a rapid and transient event, since the radiolabelled band was prominent after

Table 1 Quantification of thiophosphorylated proteins

Membrane proteins (20 μg/tube) of different origin were incubated with [^{35}S]GTP[S] (20 nM) for 60 min as described in the Experimental section. ^{35}S-radiolabelled Gβ-protein bands of different origin (see Figure 4) were cut out and subjected to liquid scintillation spectrometry. Shown are the mean values (c.p.m./mg of protein) of two different experiments (left column). Also, membrane suspensions (3 μl/tube) of different origin were incubated with [^{35}S]GTP[S] (500 nM) for 60 min as described in the Experimental section. Thereafter, samples were either directly filtered through nitrocellulose sheets or, for measurement of covalently bound radioactivity, filtered after a 15 min incubation with an equal volume of 2 $\times$ concentrated sample buffer [14]. Washed filters were analysed for radioactivity by liquid scintillation spectrometry. Shown are means $\pm$ S.D. ($n = 3$–8). Values in parentheses are the amounts of covalently bound radioactivity as a percentage of the total ^{35}S bound.

Source	$10^{-3} \times$ Radioactivity from SDS/PAGE (c.p.m./mg)	Thiophosphorylated membrane proteins (pmol/mg)
HL-60 cells	164.3	60.6 $\pm$ 13.7 (32.6%)
Human placenta	144.6	18.4 $\pm$ 2.3 (19.8%)
Human erythrocytes	9.1	6.9 $\pm$ 1.0 (6.8%)
Human platelets	16.4	5.5 $\pm$ 1.5 (6.5%)
Porcine brain	96.8	13.2 $\pm$ 2.5 (12.1%)
Porcine liver	1128.6	25.3 $\pm$ 7.0 (42.2%)
Rat brain	32.7	13.8 $\pm$ 3.4 (6.2%)
Bovine brain	220.3	15.7 $\pm$ 5.0 (10.8%)
Bovine liver	853.6	23.9 $\pm$ 9.1 (33.9%)

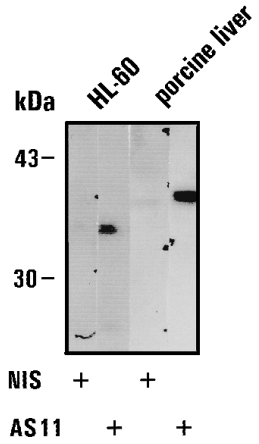

Figure 4 Immunoprecipitation of Gβ

HL-60 or porcine liver membranes (200 μg each) were phosphorylated for 1 min with [γ-^{32}P]GTP. Thereafter, proteins were solubilized and subjected to immunoprecipitation using non-immune serum (NIS) or AS11, as described in the Experimental section. After SDS/PAGE (separating gels contained 10% acrylamide), labelled proteins were visualized by autoradiography. Shown are results from one representative experiment of one SDS/polyacrylamide gel which was cut into strips and exposed to X-ray films for different periods of time because of the different intensities of radioactivity incorporated into immunoprecipitated Gβ-proteins. HL-60-derived Gβ-proteins were exposed to an X-ray film for 10 days, whereas porcine liver Gβ-proteins were exposed overnight. The molecular masses (kDa) of marker proteins are indicated.

a short time of incubation and diminished on longer duration (Figure 2, middle and bottom panels). (iii) The apparent sizes of the phosphorylated proteins varied between species but were identical for different tissues from the same species. In human specimens the phosphorylated Gβ band migrated close to unmodified human Gβ-proteins detectable with specific antibodies (AS11, AS28 and AS36, recognizing Gβ_1 and Gβ_2) at about 35 kDa. In contrast, phosphorylated proteins in other species migrated with apparently lower mobilities, corresponding to molecular masses of approx. 38 kDa (pig), 39 kDa (rat) and 42 kDa (cow). Interestingly, supplementation of SDS/PAGE with urea led to the loss of the differential mobilities of the radiolabelled protein bands between species, giving an apparent molecular mass of 41–42 kDa which was similar to the apparent size of unmodified Gβ-proteins in urea-supplemented SDS/PAGE (Figures 1 and 2). (iv) The intensity of phosphorylation varied considerably between tissues (Figure 2). The radioactive band in liver membranes exhibited the highest level of phosphate incorporation, whereas in brain and placental tissue less intense signals were visible. In contrast, only a faint band and no signal were detectable in human platelets and erythrocytes respectively. Phosphate incorporation into proteins was more effective after short times of incubation (1 min) with [γ-^{32}P]GTP than after longer times (10 min; Figure 2).

From previous studies we know that thiophosphate transfer to HL-60 cell Gβ occurs with slower kinetics than phosphate transfer. In a corresponding experiment, we thiophosphorylated cell membranes by employing [^{35}S]GTP[S] (Figure 3). Although incubation times (60 min) for thiophosphorylation were much longer than those used for phosphorylation, significant radioactivity accumulated in protein bands migrating with similar mobilities to the phosphorylated species. For instance, thiophosphorylated human Gβ isoforms migrated faster than their porcine counterparts on separating gels containing 10% acrylamide (Figure 3, upper panel). When urea was added to the separating gels, however, thiophosphorylated Gβ isoforms from both species migrated similarly at approx. 41 kDa (Figure 3, lower panel). For better evaluation, labelled bands were cut out and counted for radioactivity (Table 1). Platelets and erythrocytes showed negligible incorporation of radioactivity, whereas liver proteins most effectively incorporated [^{35}S]thiophosphate. Interestingly, rat brain membranes appeared to be less thiophosphorylated than phosphorylated under these experimental conditions.

In order to obtain a rough estimate of the amount of Gβ thiophosphorylation in different tissues, we analysed membrane proteins for their ability to bind [^{35}S]GTP[S] and quantified protein thiophosphorylation by using a filtration assay as described previously [7]. Liver membranes showed the highest level of thiophosphorylation regardless of whether the results are given relatively as a percentage of total [^{35}S]GTP[S] binding or as absolute values (Table 1). Thiophosphorylation of liver proteins was, therefore, significantly greater than that of brain membranes of the respective species. Human placental membranes showed a concentration of 18.4 $\pm$ 2.3 pmol of thiophosphorylated protein/mg of protein, which was significantly higher than the concentrations observed in human erythrocytes and platelets.

Identification of the radiolabelled protein and its modified amino acid

Previous experiments have shown that phosphorylation or thiophosphorylation of human HL-60 cell membranes or rod outer segment membranes of bovine origin resulted in specific incorporation of radioactivity into Gβ [4,6]. Therefore it was likely that the radioactive bands observed on SDS/PAGE after labelling of membranes of other species also represented phosphorylated Gβ. In order to verify this assumption, we immunoprecipitated phosphorylated proteins from human HL-60 and porcine liver cell membranes (Figure 4). In both cases, the anti-Gβ specific antibody AS11 precipitated a single band which differed in apparent molecular mass to the same extent as the radioactive bands shown in Figure 2. Immunoprecipitated porcine Gβ had much more radioactivity incorporated than the

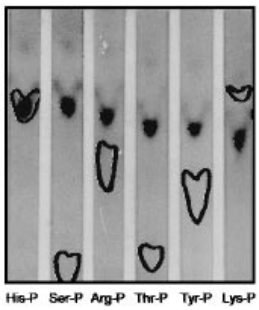

Figure 5 Identification of phosphohistidine in Gβ

HL-60 membranes (2.3 mg of protein) were incubated with [γ-^{32}P]GTP (20 nM) for 3 min at 30 °C. After SDS/PAGE fractionation, proteins were transferred to nitrocellulose by Western blotting and radioactivity was visualized by autoradiography. A 35 kDa radiolabelled protein band was excised and hydrolysed with KOH, as described in the Experimental section. Subsequently samples were subjected to TLC. Developed TLC plates were exposed to X-ray films and phosphoprotein standards were visualized by incubation with ninhydrin. Shown is the autoradiogram. The positions of ninhydrin-stained standard phosphoproteins are indicated.

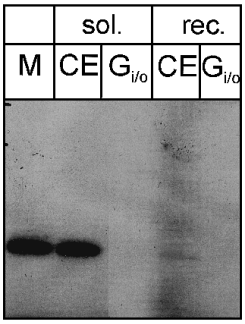

Figure 6 Phosphorylation of proteins

Membrane proteins (M) and solubilized (sol.) or phospholipid-reconstituted (rec.) cholate-extracted proteins (CE) or purified $G_{i/o}$ proteins of bovine brain origin were phosphorylated with [γ-^{32}P]GTP (20 nM) as described in the Experimental section. Proteins were resolved on an SDS gel containing 10% acrylamide. Shown is the autoradiogram of the exposed gel.

human counterpart isolated from HL-60 membranes (for details, see legend to Figure 4). Hence it is most likely that the radioactive bands of 35–42 kDa observed in cell membranes from four species are indeed phosphorylated Gβ subunits.

Next we analysed the identity of the Gβ modification, by subjecting phosphorylated HL-60 Gβ to phosphoamino acid analysis. Radioactivity derived from Gβ co-migrated only with phosphohistidine (Figure 5), confirming our previous assumption that a histidine is the target amino acid [6].

Having identified the target protein and its modified amino acid, we sought to extract Gβ-phosphorylating activity from cell membranes. Various samples of bovine brain origin obtained from a routine G_i/G_o purification [11] were examined for their ability to phosphorylate Gβ (Figure 6). By using cholate extracts it was possible to phosphorylate a protein co-migrating with [^{32}P]Gβ of bovine brain membranes, but the signal was weaker than in native membranes. In contrast, purified mixtures of G_i/G_o-proteins which consisted of all three G_i and three G_o subtypes, including the G_1 and G_2 isoforms [10], exhibited no Gβ-phosphorylating activity regardless of whether the solubilized or liposome-reconstituted G-protein preparation was phosphorylated (Figure 6).

DISCUSSION

Post-translational modifications of native membrane-bound mammalian Gβ subunits were first shown for $G\beta_1$ in rod outer segment membranes of bovine retinae, and later for an HL-60 Gβ subunit. In both cases, Gβ-proteins underwent phosphorylation [4,6]. This Gβ phosphorylation is part of a phosphate transfer reaction resulting in rapid and transient phosphorylation of membrane-bound GDP [7]. Consequently, we have speculated whether Gβ phosphorylation represents an alternative route of G-protein activation that avoids the GDP–GTP exchange reaction on Gα. Others have suggested that phosphorylated G$\beta\gamma$ may represent the active state of the dissociated G$\beta\gamma$ complex, analogous to the GTP-bound Gα subunit [20]. However, nothing was known about the generality of this phenomenon. We therefore studied cell membranes from different tissues and species for their ability to undergo this high-energy-phosphate transfer reaction. Although the present study demonstrates that Gβ phosphorylation is a general mechanism occurring in the tissues of all species tested, marked differences between species and tissues were obvious. In contrast to human placental tissue and HL-60 cells, human platelets and erythrocytes exhibited very low, if any, Gβ phosphorylation, although significant amounts of $G\beta_1$ and $G\beta_2$ immunoreactivity were detectable. This points to the possibility that modification of Gβ requires a cofactor that is only weakly expressed or lacking in some cell membranes, e.g. those of erythrocytes. Further support for this assumption comes from the observation that Gβ is easily phosphorylated in native cell membranes and detergent extracts, whereas purified and liposome-reconstituted G-proteins are resistant to this modification.

This study has unveiled tissue-dependent differences in the efficacy of Gβ phosphorylation. Brain membranes exhibited less Gβ phosphorylation than others, e.g. liver cell membranes, although they contained the highest amounts of $G\beta_1$ and $G\beta_2$ immunoreactivity. These differences may be caused by quantitative and/or qualitative differences in the expression of the predicted cofactor in various cells and tissues, or they may simply be based on Gβ heterogeneity. For the latter reason, one has to keep in mind that only expression of $G\beta_1$ and $G\beta_2$ proteins was confirmed. In liver membranes the amount of covalently bound thiophosphate reached approx. 40 % of total [^{35}S]GTP[S] binding. Considering the data obtained from the GTP[S] binding assay, they reflect on the one hand association of GTP[S] with Gα and other GTPases, including monomeric G-proteins, and on the other hand thiophosphorylation of Gβ. It is conceivable that the latter reaction is almost quantitative in cases where the cofactor is highly expressed.

During the course of our study, a second notable feature of Gβ phosphorylation heterogeneity became obvious, i.e. differences in the apparent mobilities of the phosphorylated Gβ subunits of different species (Figure 2). The human phosphoprotein was previously demonstrated to be Gβ [6], and we have now confirmed the identity of the porcine phosphoprotein as Gβ (Figure 4). Although antiserum AS11 was raised against a peptide sequence specific for $G\beta_1$, this antibody clearly also detects $G\beta_2$, $G\beta_3$ and $G\beta_4$ ([13]; B. Nürnberg, K. Spicher, C. Hartenedk and G. Schultz, unpublished work). Therefore it remains to be clarified whether the human and porcine phosphorylated proteins differ in their primary amino acid sequence, whether they represent different Gβ subtypes or whether the difference in apparent mobility is caused by other factors, e.g. differences in the stoichiometry of phosphorylation. Interestingly, phosphorylated Gβ-proteins of rat and bovine origin also exhibited different electrophoretic mobilities. Regardless of the identity of the Gβ

isoform, the results suggest that only one isoform undergoes phosphorylation. It appears that one single isoform is expressed in different tissues of the same species, since only one radioactive band was visible in different SDS/PAGE systems (Figure 2). Previously we speculated about the target amino acid in Gβ for phosphorylation, based on indirect evidence [6]. Using HL-60-derived Gβ we have now confirmed that a histidine is the amino acid that is modified (Figure 5). This finding is in accordance with the chemical features of the modification, e.g. high energy bonds that are stable on treatment with NaOH but are sensitive to treatment with heat, HCl and hydroxylamine.

Taken together, our results show that Gβ subunits are phosphorylated on a histidine residue, presumably catalysed by a histidine kinase. Histidine kinases are known to be present in mammals [16,21–23]. We are currently attempting to isolate this cofactor.

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