Activation of Heterotrimeric G Proteins by a High Energy Phosphate Transfer via Nucleoside Diphosphate Kinase (NDPK) B and Gβ Subunits

COMPLEX FORMATION OF NDPK B WITH Gβγ DIMERS AND PHOSPHORYLATION OF His-266 IN Gβ*

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G protein βγ dimers can be phosphorylated in membranes from various tissues by GTP at a histidine residue in the β subunit. The phosphate is high energetic and can be transferred onto GDP leading to formation of GTP. Purified Gβγ dimers do not display autophosphorylation, indicating the involvement of a separate protein kinase. We therefore enriched the Gβ-phosphorylating activity present in preparations of the retinal G protein transducin and in partially purified Goα proteins from bovine brain. Immunoblots, autophosphorylation, and enzymatic activity measurements demonstrated enriched nucleoside diphosphate kinase (NDPK) B in both preparations, together with residual Gβγ dimers. In the retinal NDPK B-enriched fractions, a Gβ-specific antiserum co-precipitated phosphorylated NDPK B, and an antiserum against the human NDPK co-precipitated phosphorylated Gβγ. In addition, the NDPK-containing fractions from bovine brain reconstituted the phosphorylation of purified Gβγ. For identification of the phosphorylated histidine residue, bovine brain Gβγ and Gγγ were thiophosphorylated with guanosine 5′-O-(3-[35S]thio)triphosphate, followed by digestion with endoproteinase Glu-C and trypsin, separation of the resulting peptides by gel electrophoresis and high pressure liquid chromatography, respectively, and sequencing of the radioactive peptides. The sequence information produced by both methods identified specific labeled fragments of bovine Gβγ that overlapped in the heptapeptide, Leu-Met-Thr-Tyr-Ser-His-Asp (amino acids 261–267). We conclude that NDPK B forms complexes with Gβγ dimers and contributes to G protein activation by increasing the high energetic phosphate transfer onto GDP via intermediately phosphorylated His-266 in Gβ1 subunits.

Heterotrimeric G proteins play a pivotal role in many signal transduction pathways in eukaryotic cells. They consist of a guanine nucleotide-binding α subunit (40–52 kDa), a β subunit (33–43 kDa), and a γ subunit (6–10 kDa). The latter two act as a functional unit and only dissociate upon denaturation. Both Ga and Gβγ are required for receptor-induced G protein activation and can trigger effector functions (for reviews see Refs. 1–3). Heterotrimeric G proteins are activated by a GDP/GTP exchange catalyzed by G protein-coupled receptors. Furthermore, we and other laboratories provided evidence that photophosphoryl transfer reactions can participate in G protein activation in vitro by formation of GTP. There is ambivalent evidence that nucleoside diphosphate kinase (NDPK) contributes to G protein activation by replenishment of GTP from ATP and GDP (for reviews see Refs. 4–6). Hypotheses suggesting a direct in situ phosphorylation of GDP bound to Ga and monomeric G proteins (7–9) are most likely based on artifacts. Also complex formation of NDPK with G proteins and channeling of NDPK-formed GTP into Ga (10, 11) have not yet been proven beyond doubt.

A photophosphorylation reaction that uses Gβ subunits as phosphorylated intermediates has been observed in various tissues (12–16). In this reaction, the γ-thio)phosphate group of GTP or its analog GTPγS is transferred onto a histidine residue of Gβ. Apparently, a membrane-bound, so far unknown co-factor is required to achieve this phosphorylation (14, 15). Out of the labile high energy phosphoamidate bond, the phosphate can be retransferred onto GDP to form GTP, which then can activate Gα1 and Gαi proteins and thus regulates, for example, adenyl cyclase activity (17). Nevertheless, the exact significance of this photophosphorylation reaction remains elusive.

The purpose of this study was to test the hypothesis that NDPK may represent the unknown co-factor and contributes to the phosphate transfer via Gβ. We report here that by the attempts to purify the co-factor from the retinal G protein transducin (Gα1) or bovine brain membranes, we specifically enriched the NDPK B isoform. We will further provide evidence for a complex formation of Gβγ with NDPK B and for a specific phosphorylation of His-266 of Gβ1 in this complex.

EXPERIMENTAL PROCEDURES

Preparation of Rod Outer Segment Membranes, Purification of NDPK, Purification of Gα, and Its Subunits, and Resolution of the Gβ-Phosphorylating Activity from Gα1—Rod outer segment (ROS) membranes were prepared from illuminated bovine retina according to

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1 The abbreviations used are: NDPK, nucleoside diphosphate kinase; GTPγS, guanosine 5′-O-(3-thio)triphosphate; GppNHp, guanosine 5′-O-(β,γ-imino)triphosphate; ROS, rod outer segment; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; HPLC, high pressure liquid chromatography; Mm, molecular mass.
G Protein Activation by Phosphate Transfer I

Purification of \( \alpha \)-proteins, \( \beta \gamma \)-, and the \( \beta \gamma \)-Phosphorylating Activity from Bovine Brain Membranes—Membranes from bovine brain were prepared as described (20). Proteins were solubilized from membranes (4 g of protein) by stirring for 1 h at 4 °C in 800 ml of TEM buffer (20 mM Tris-HCl, pH 8, 1 mM EDTA, 20 mM 2-mercaptoethanol) containing 1% (w/v) 1-octyl-1-\( \beta \)-thioglucopyranoside (Biomol, Hamburg, Germany). After centrifugation for 40 min at 110,000 × g, ethylene glycol was added at 20% (v/v) of the supernatant to the pellet containing 20% of the extract, i.e., ~800 mg of protein, were used for three subsequent steps of liquid chromatography, carried out at 4 °C, using a fast protein liquid chromatography device. Elution was monitored by continuous measurement of absorbance at 280 nm. The extract was first loaded onto a column (10 × 13 cm) containing 1 liter of DEAA- Sepharose (Amersham Biosciences) at a flow rate of 0.5 ml/min. After washing with TEMEC (TEM buffer containing additionally 30% ethylene glycol and 0.9% (w/v) sodium cholate), elution was performed with a linear gradient of NaCl (0–800 mM, volume 2.2 liters). Fractions of 12 ml were collected. \( \alpha \)-proteins eluted from the column at about 450 mM NaCl were further purified and separated into their subunits as described (20). Fractions of 2 ml of thiophosphorylated \( \beta \gamma \) subunits were identified in the phosphorylation assay (see below), using 0.3 μg of bovine brain \( \gamma \) subunit as substrate. Positive fractions eluting at 220–270 mM NaCl were pooled and concentrated by pressure filtration using a PM 10 membrane. This pool (about 35 mg of protein) was then loaded onto a column (3 × 14 cm) containing 100 ml of hydroxyapatite (E. Merck, Darmstadt, Germany) at 5 ml/min. The column was washed with TEMEC. Elution of the cation exchange column was performed with a linear gradient of NaCl (0–1 x, volume 80 ml) in HEMEC. Fractions of 2 ml were collected. Positive fractions eluting at 250–290 ml of HEMEC were concentrated by centrifugation in a Centri-200 device (Amicon). Chromatography media were regenerated according to the respective manufacturer’s recommendations.

Phosphorylation and Thiophosphorylation of \( \beta \gamma \) Subunits and NDPK—The indicated amounts of bovine brain or transducin \( \gamma \) subunits were phosphorylated with 10 mM \( [\gamma ^{32}P] \)GTP (PerkinElmer Life Sciences) for the indicated periods of time at 30 °C in a reaction buffer containing 50 mM triethanolamine hydrochloride, pH 7.4, 150 mM NaCl, 2 mM MgCl\(_2\), 1 mM EDTA, and 1 mM dithiothreitol with a total volume of 20 μl. For thiophosphorylation of \( \gamma \) subunits, 20 μl \( [\gamma ^{32}P] \)GTP/\( \gamma \)-S was used, and incubation was performed at 30 °C with a total reaction volume of 125 μl. The reaction was terminated by the addition of 1 ml of each fraction. The sequence of the peptide was determined by Edman degradation in an Applied Biosystems, Inc. (Foster City, CA) model 476A protein sequencer.

Tryptic Digest of Thiophosphorylated \( \gamma \)-res, Resolution, and Sequencing of Peptides—Unmodified \( \gamma \)-res (5 μg) was thiophosphorylated with 100 μM \( [\gamma ^{32}P] \)GTP/\( \gamma \)-S and 1 μg of NDPK B-enriched co-factor as described above, in a total volume of 100 μl. The reaction was terminated by addition of 50 μl of 3-fold concentrated SDS-PAGE sample buffer and 10 μg of endoproteinase Glu-C (Roche Molecular Biologicals). Proteins were digested for 3 h at 37 °C, and the proteolytic fragments were separated on a 3%–20% Tricine-based system (23). After electrotransfer onto a nitrocellulose membrane, the labeled fragment was identified by autoradiography and excised. The peptide was eluted, and its amino acid sequence was determined by automated Edman degradation in an Applied Biosystems, Inc. (Foster City, CA) model 476A protein sequencer.

RESULTS

\( \beta \gamma \)-Phosphorylating Activity in \( G \), and Complex Formation of \( \beta \gamma \) with NDPK—The \( \beta \) subunits of the retinal G protein \( G \) can be transiently (thiophosphorylated by GTP or GTP/\( \gamma \)-S and

Western Blot Analysis—Proteins were separated by SDS-PAGE (10–12% polyacrylamide in the resolving gel or modified gel) (23) and electrophoresed to nitrocellulose membranes (Schleicher & Schuell). Blots were washed with TBS (10 mM Tris-HCl, pH 7.4, 154 mM NaCl), incubated overnight at 4 °C with 3% skim milk in TTBS (TBS + 0.05% Tween 20), washed with TTBS, and incubated with the respective antisera (anti-\( G \) (T-20; 1:1000) (Santa Cruz Biotechnology, Inc.), anti-NDPK (C-20; 1:100) (Santa Cruz Biotechnology, Inc.), anti-\( \alpha \) (T-20; 1:100) (kind gift of Dr. Ioan Lascu), and rabbit IgG anti-human NDPK B (1:500) (kind gift of Dr. Ioan Lascu)) for 3 h at room temperature. Horse radish peroxidase-conjugated anti-rabbit IgG antibody (1:1000) was used as secondary antibody. Specific bands were detected with the enhanced chemiluminescence system (Amersham Biosciences).

Immunoprecipitation—The pooled fractions (60 μl) from the hydroxyapatite column, containing the peak activity of NDPK from \( G \), were phosphorylated in a final volume of 80 μl at 30 °C for 7 min. The reaction was terminated by placing the mixture on ice and adding an equal volume of 50 mM EDTA, pH 7.4. Precipitation buffer (280 μl), containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 25 mM EDTA, 1 mM dithiothreitol, 1 mM NaF, and 0.2 μM phenylmethylsulfonyl fluoride, was added, followed by the addition of 10 μl of the same buffer containing protein A-Sepharose beads. After incubation on ice for 20 min and centrifugation (26,000 × g, 15 min), the clear supernatant was taken and supplemented with 0.6 μg of anti-\( G \) (T-20) or 4 μg of anti-NDPK (C-20) antiserum and 5 μg of protein A-Sepharose beads. The mixture was gently shaken for 2 h at 4 °C. Protein A-Sepharose beads were pelleted and washed three times with precipitation buffer containing 300 mM NaCl. Bound proteins were eluted from the protein A-Sepharose beads by adding 50 μl of SDS buffer. The samples were kept at room temperature for at least 1 h before loading onto a 10% polyacrylamide gel.

Treatment of \( G \) with Diethyl Pyrocarbonate—Purified \( G \) (30 μg) was incubated for 10 min at room temperature in a buffer (100 mM) containing the hydroxyapatite column (1.5 ml) and 100 mM diethyl pyrocarbonate. The reaction was terminated by the addition of 25 mM EDTA. Free diethyl pyrocarbonate and EDTA were subsequently removed by repeated buffer exchange into 20 mM Tris-HCl, pH 8, 1 mM EDTA, 20 mM 2-mercaptoethanol using a Microcon-10 device (Amicon).

Endoproteinase Glu-C Digest of Thiophosphorylated \( \gamma \)-res, Purification, and Sequencing of Peptides—Bovine brain \( \gamma \) (15 μg of protein) was thiophosphorylated with 100 μM \( [\gamma ^{32}P] \)GTP/\( \gamma \)-S and 1 μg of NDPK B-enriched co-factor as described above, in a total volume of 100 μl. The reaction was terminated by addition of 50 μl of 3-fold concentrated SDS-PAGE sample buffer and 10 μg of endoproteinase Glu-C (Roche Molecular Biologicals). Proteins were digested for 3 h at 37 °C, and the proteolytic fragments were separated on a 3%–20% Tricine-based system (23). After electrotransfer onto a nitrocellulose membrane, the labeled fragment was identified by autoradiography and excised. The peptide was eluted, and its amino acid sequence was determined by automated Edman degradation in an Applied Biosystems, Inc. (Foster City, CA) model 476A protein sequencer.

Phosphorylation and Thiophosphorylation of \( \beta \gamma \) Subunits and NDPK—The indicated amounts of bovine brain or transducin \( \gamma \) subunits were phosphorylated with 10 mM \( [\gamma ^{32}P] \)GTP (PerkinElmer Life Sciences) for the indicated periods of time at 30 °C in a reaction buffer containing 50 mM triethanolamine hydrochloride, pH 7.4, 150 mM NaCl, 2 mM MgCl\(_2\), 1 mM EDTA, and 1 mM dithiothreitol with a total volume of 20 μl. For thiophosphorylation of \( \gamma \) subunits, 20 μl \( [\gamma ^{32}P] \)GTP/\( \gamma \)-S was used, and incubation was performed at 30 °C with a total reaction volume of 125 μl. The reaction was terminated by the addition of 1 ml of each fraction. The sequence of the peptide was determined by Edman degradation and analysis of GTP formation were performed essentially as described (25).
an enzymatic activity present in ROS membranes (12). More recently, it was demonstrated that soluble preparations of Gt contain NDPK activity and that GTP phosphorylates a 36-kDa protein, most likely Gtβ (22). We therefore attempted to identify the putative co-factor present in the Gt preparations and investigated whether NDPK is involved in this reaction. For this purpose, Gt was eluted from ROS membranes with 100 μM of the stable GTP analog, GppNHP, which does not modify Gtβγ (12). Gtβγ and Gtα can be resolved from each other by affinity chromatography on Blue-Sepharose (12, 22). Whereas Gtβγ has little affinity to the matrix, elution of Gtα requires high salt concentrations (500 mM KCl). Single protein bands at the apparent molecular weights of Gtβγ and Gtα were detected by SDS-PAGE and Coomassie Blue staining (not shown) of the fractions from the first and second peak, respectively. In search for the Gtβ-phosphorylating activity, the fractions were subjected to phosphorylation with [γ-32P]GTP. In the Gtα-containing fraction, two phosphorylated proteins (M sub 18 and 36 kDa; see Fig. 1) were detected. Measurement of NDPK activity revealed a formation of 0.2 nmol GTP per mg of protein per min in this fraction. In line with data reported previously (12, 22), no phosphorylated proteins and no NDPK activity was detected in the Gtβγ-containing fractions. To separate the two phosphorylated proteins from Gtα, 1 mg of protein from the second peak was applied onto a hydroxyapatite column, and proteins were eluted with increasing concentrations of potassium phosphate (0–400 mM). Gtα eluted from the column in a single peak at about 10 mM phosphate (fractions 23–26; see Fig. 1A). No NDPK activity and no phosphorylation by [γ-32P]GTP were detected in the Gtα peak. The NDPK activity eluted from the column at ~250 mM phosphate (Fig. 1B). As shown in the inset of Fig. 1B, this fraction contained both the 18- and 36-kDa proteins phosphorylated by [γ-32P]GTP. Immunoblot analysis showed that this fraction contained NDPK protein, Gtβγ, and traces of Gtα.

To identify the two proteins phosphorylated by [γ-32P]GTP, we immunoprecipitated Gtβ and NDPK with specific antisera after phosphorylation. Identical amounts of IgG were used as controls. As shown in Fig. 2, the NDPK-specific antiserum precipitated NDPK as a doublet migrating at 18–20 kDa and an additional phosphoprotein at 36 kDa. The Gtβ-specific antiserum precipitated phosphorylated Gtβ (36 kDa) and an additional phosphoprotein at 18–20 kDa. Some minor phosphorylated bands in the range of 28 to 30 kDa most likely represent degradation products of phosphorylated Gtβ.

Enrichment of the Gtβ-Phosphorylating Activity from Bovine Brain Membranes and Reconstitution of Gtβ Phosphorylation—As Gtβ differs from other G proteins by its solubility without detergent, we attempted to purify the Gtβ-phosphorylating activity from another tissue. Because heterotrimeric G proteins are abundant in bovine brain membranes, and the extent of Gtβ phosphorylation is rather high compared with membranes of other tissues available in sufficient amounts (15), we first passed a detergent extract from bovine brain membranes over a DEAE column. G proteins that elute from the column at about 450 mM NaCl could no longer be phosphorylated with [γ-32P]GTP (15) (not shown). Apparently, the co-factor that promotes phosphorylation had been separated from the majority of G proteins. Addition of the fraction eluting at about 250 mM NaCl to the G protein-containing fraction or purified bovine brain Gtβγ reconstituted the phosphorylation. The fraction that promoted Gtβ phosphorylation was further purified by hydroxyapatite chromatography, followed by cation exchange chromatography using an EMD SO3 650(S) column. After each step, positive fractions were identified by their potential to phosphorylate Gtβ. Equal amounts of protein (1 μg) were used after each step to reconstitute the phosphorylation of purified bovine brain Gtβγ (0.5 μg). Phosphorylated Gtβ subunits were excised from SDS-PAGE gels, and the amount of radioactivity was detected by liquid scintillation counting, to estimate the specific activity after each purification step. The data are summa-
rized in Table I. A small decrease in specific activity after the EMD SO₃ cation exchange column was noted. However, the chromatogram showed that 97% of total protein could be removed during this step. Thus, the decrease in specific activity might have been because of decaying enzymatic activity in the diluted protein fraction during longer storage at 4 °C. SDS-PAGE and silver staining revealed that the fractions contained four to five faint protein bands in the molecular range from 20 to 50 kDa (not shown). Similar to the Gβ-phosphorylating activity prepared from Gt, NDPK activity (1.8 nmol of GTP formed per mg protein per min) and NDPK protein (immunoblot, autophosphorylation) could be detected (Fig. 3A). To test whether this preparation is able to reconstitute the phosphorylation of bovine Gβγ, increasing amounts of the EMD SO₃-650(S) pool (0.04–0.4 μg of protein) were phosphorylated with [γ-³²P]GTP in the absence and presence of purified Gβγ (0.5 μg) for 5 min at 30 °C (Fig. 3B). Conversely, the phosphorylation of increasing amounts of purified bovine brain Gβγ dimers promoted by a constant quantity of partially purified factor (200 ng of protein) was studied (Fig. 3C). The phosphorylation of Gβ increased with the amount of added co-factor fraction or Gβγ dimer. Maximal phosphorylation was observed with 1–3 μg of Gβγ dimer. Similar data were obtained when G1,βγ was used as substrate (Fig. 3D). Previous results showed that pretreatment of membranes with diethyl pyrocarbonate prevents the phosphorylation of Gβ because of the ethoxycarbonylation of histidine residues (13, 24). Similarly, G1,βγ pretreated with diethyl pyrocarbonate (10 mM) prior to phosphorylation with the co-factor-containing fraction exhibited no increase in phosphorylation of the ethoxycarbonylated Gβγ (Fig. 3D). In addition, phosphorylated Gβ was sensitive to treatment with hydroxylamine (data not shown) that cleaves phosphohistidine (25). As shown in Fig. 3E, the co-factor-containing fraction also could be identified as the thio phosphorilation of Gβ by [³²S]GTP·S. When higher amounts of the co-factor pool were used, the phosphorylated Gβ was detected also in the absence of added Gβγ (Fig. 3, A, B, and D). Indeed, small amounts of Gβ could be identified in the co-factor pool by Western blot analysis (Fig. 3A).

**Table I**

Summary of the enrichment of the Gβ-phosphorylating activity from bovine brain membranes

| Purification step | Volume (ml) | Protein concentration (μg/μl) | Total protein content (μg) | cpm/μg protein | Specific activity (cpm/μg protein) |
|------------------|-------------|------------------------------|----------------------------|----------------|-----------------------------------|
| Detergent extract | 930         | 889                          | 826.8                      | 82.8           | 1                                 |
| DEAE             | 170         | 203                          | 34.5                       | 725.9          | 8.8                               |
| Hydroxypatitide  | 36          | 167                          | 6.3                        | 1823.7         | 22.0                              |
| EMD SO₃          | 4.5         | 42                            | 189                        | 1506.8         | 18.2                              |

**Fig. 3. Reconstitution of the Gβ (thio)phosphorylation of Gβγ.** A, the co-factor required for Gβ phosphorylation in solubilized bovine brain membranes was enriched by subsequent anion exchange, hydroxyapatite, and cation exchange chromatography as described under "Experimental Procedures." The fractions obtained from the cation exchange column at 350–380 mM NaCl were pooled. After phosphorylation with [γ-³²P]GTP for 5 min at 30 °C, 800 ng of protein were subjected to SDS-PAGE. Phosphorylated proteins were visualized by autoradiography. Gβ and NDPK were detected by Western blotting with specific antibodies. B, increasing amounts (40–400 ng of protein) of the co-factor pool (CF) were phosphorylated in the absence (−) and presence (+) of 0.5 μg of purified bovine brain Gβγ for 5 min at 30 °C. C, increasing amounts of Gβγ (10–3000 ng) were phosphorylated in the presence of 200 ng of the cation exchange fraction. D, Gβγ was treated with 10 mM diethyl pyrocarbonate as described under "Experimental Procedures." Thereafter, phosphorylation of Gβ by [γ-³²P]GTP was determined with the cation exchange fraction in the absence (Control) and presence of 2 μg untreated (G,Gβγ) or diethyl pyrocarbonate-treated Gβγ (Gβγ/DEPC). E, 200 ng of the cation exchange fraction were thio phosphorylated with 20 nM [³²S]GTP·S in the absence (Control) and presence of 1 μg of purified bovine brain Gβγ for 30 min at 30 °C. Autoradiographs after SDS-PAGE are shown.
IgG anti-human NDPK A (immunoblot analysis with the NDPK isoform-specific antisera, rabbit antiserum, only NDPK B was detected in the GNDPK A antiserum was more sensitive than the anti-NDPK B bovine ROS served as positive controls. Although the anti-by Dr. Ioan Lascu and purified NDPK from the cytosol of fied recombinant human NDPK A and B (also kindly provided provided by Dr. Ioan Lascu, Bordeaux, France) (Fig. 4). Puri-

Western blot analysis with subtype-specific antisera (kindly

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A

rec Cytosolic NDPK Gβγ-NDPK Gβγ-NDPK

NDPK A

B

rec Cytosolic NDPK Gβγ-NDPK Gβγ-NDPK

NDPK B

Fig. 4. Immunoblot analysis of the NDPK isoforms in the Gβ-

phosphorylating activities from bovine retina and bovine brain.

Purified recombinant human NDPK A (A, 10 ng; rec) or NDPK B (B, 10 ng; rec), 500 ng of NDPK purified from the cytosol of bovine ROS, the Gβ-phosphorylating activity from bovine retina (30 μl; Gβγ-NDPK), and bovine brain (1 μg; Gβγ-NDPK) were subjected to SDS-PAGE and immunoblot analysis with the NDPK isoform-specific antisera, rabbit IgG anti-human NDPK A (A) or rabbit IgG anti-human NDPK B (B). Duplicate values are shown.

retina ROS (19) and other tissues (5, 6). We therefore investi-
gated whether one or both forms had been enriched during the purification procedures for the Gβ-phosphorylating activity by Western blot analysis with subtype-specific antisera (kindly provided by Dr. Ioan Lascu, Bordeaux, France) (Fig. 4). Purified recombinant human NDPK A and B (also kindly provided by Dr. Ioan Lascu) and purified NDPK from the cytosol of bovine ROS served as positive controls. Although the anti-

NDPK A antiserum was more sensitive than the anti-NDPK B antiserum, only NDPK B was detected in the Gβ-phosphorylating fractions obtained from Gt and bovine brain membranes.

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-endoproteinase Glu-C would produce several peptides in the range of 1000 to 13,500 kDa. Each contains not more than three histidine residues. We therefore asked whether we could detect a labeled phosphopeptide after in gel digest with endoproteinase Glu-C and separation of the peptides by high resolution SDS-PAGE (19). As shown in Fig. 5A, endoproteinase Glu-C digest of phosphorylated bovine brain Gβγ produced a single labeled peptide (Mn – 9 kDa). The same peptide is recognized by the anti-Gβ (T-20) antibody (Fig. 5B). To identify the phosphopeptide, 15 μg of bovine brain Gβγ were thiophosphorylated with 100 nM [35S]GTPγS and digested with endoproteinase Glu-C. After SDS-PAGE and electrophor-onto nitrocellulose, the labeled peptide was identified by autoradiography and excised from the blot. The peptide was eluted and sequenced by Edman degradation (26). The experiment was repeated twice. The first run produced the sequence LMYXYYXXDII. In the second run, the 14-amino acid sequence, LMTYSHDNICGIT, could be identified. This sequence corre-

on the N terminus of a peptide (amino acid 261–340; Mn 8657.71) that results from endoproteinase Glu-C digest of Gβ1, and contains two histidine residues, His-266 and His-311.

Although Gβγ complexed with different Gγ subunits is abun-
dant in bovine brain Gβγ preparations, at least Gβ2γ4 dimers are similarly present (27). We therefore used the well defined Gβγ4, i.e. Gβγ4, for further analysis. Unmodified Gβγ4 was thiophosphorylated with 100 nM [35S]GTPγS and the co-factor from bovine brain (see Fig. 3) and added to 10 μg of unlabeled thiophosphorylated Gβγ4. The mixture was digested overnight with trypsin, the peptides were separated by HPLC on a C18 reverse phase HPLC column, and peak fractions were collected (Fig. 6A). An aliquot of each fraction (3 μl) was counted for radioactivity. As shown in Fig. 6B, the labeled peptide eluted from the column in a sharp peak at fraction 22. The analysis of this fraction by Edman degradation revealed the sequence ADQELMXYSHD. It corresponds to the N terminus of an ex-
expected tryptic fragment (amino acid 257–280; Mw 2659.96) that overlaps with the labeled fragment of the endoproteinase Glu-C digest. Most important, it contains only His-266 of Gβ1.

**DISCUSSION**

Meanwhile, several laboratories have reported the intermediate formation of a high energy phosphoamidate bond on a histidine residue of Gβ subunits (12–17) that enables the formation of GTP by transferring this phosphate onto GDP. The phosphorylation of Gβ requires a co-factor that acts as a histidine kinase (15, 16, 28). In addition, early studies on an NDPK-like activity leading to formation of GTP from ATP and GDP in retinal extracts from frogs associated this so called GDP kinase activity with the G3p/GDP/GTP interaction with Gβ from phosphen (29). In this study, we obtained several lines of evidence for a complex formation of the NDPK B isoform with G3pβ dimers. First, NDPK B and G3pβ were co-purified from both transducin and solubilized bovine brain membranes with different purification protocols. Apparently, the biochemical properties of the NDPK determined the purification of the complex. In contrast to Gβ2, NDPK binds tightly to Blue-Sepharose, and this property can be used to purify NDPK (19). Recently, the NDPK of Drosophila melanogaster was purified by a combination of anion exchange and hydroxyapatite chromatography (30). Gβ1γ2 and G3pβ exhibit only a weak interaction with hydroxylapatite and elute from the matrix at much lower concentrations of phosphate (31, 32). Second, a Gβ-specific antiserum co-immunoprecipitated NDPK, and conversely, an NDPK-specific antiserum co-immunoprecipitated Gβ from the retinal preparation. In agreement with our data more indirect evidences for an interaction of NDPK B with G3 have been described previously by others (11, 22). The presence of NDPK B and G3pβ or heterotrimeric G proteins, however, is apparently not sufficient to obtain the complex formation between Gβp and NDPK B to reconstitute the phosphorylation of Gβ. Most likely, another protein is required as scaffold for that complex. Recently, it was demonstrated that NDPK forms a complex with phosine, Eps 15, and dynamin I, a GTPase that plays a critical role in endocytosis (33). Within this complex, NDPK interacts with dynamin I through a proline-rich domain, whereas its interaction with phosine is ill defined. The data, however, indicate that complexes of NDPK with multiple proteins occur as mechanisms for local GTP replenishment within cells.

The question arises whether NDPK B within the complex is the histidine kinase required for the phosphorylation of Gβ. Protein kinase activity for NDPK has been described. Serine and threonine phosphorylation by NDPK was found on histone 2b, casein, and ovalbumin (34, 35). Moreover, NDPK phosphorylates the catalytic histidine residue in ATP citrate lyase (36), and other reports (37–39) indicate that NDPK is the phosphate donor for the phosphorylation of a histidine in annexin I. Most recently, Kowluru (28) reported that the phosphorylation of Gβ and histone 4 was largely increased by mastoparan, a known activator of NDPK and G proteins (40, 41). In accordance with the presumed histidine kinase activity of NDPK B, an increase in Gβ phosphorylation was observed in membranes of H10 cells overexpressing wild-type NDPK B but not its catalytically inactive mutant H118N (42).

The NDPK B-enriched fraction obtained from bovine brain reconstituted the phosphorylation and thiophosphorylation of Gβ by GTP and GTPγS, respectively. As observed before in membranes (13, 15, 16), phosphorylated Gβ was sensitive to hydroxylamine cleavage (25) and was prevented by ethoxycarbonylation with diethyl pyrocarbonate (23). Thus, all our results in the reconstituted systems are in agreement with earlier results obtained on the (thio)phosphorylation of a histidine in Gβ. The data obtained from proteolytic digest of the thiophosphorylated bovine brain Gβ2γ2 revealed that His-266 of Gβ1 is the phosphorylated residue in Gβ. As shown in Fig. 7, the imidazolyl side chain of His-266 is freely accessible on the surface of the heterotrimeric G protein and can therefore be the target of protein phosphorylation. The seven other histidines are part of the Gβ propeller structure and thus are unlikely accessible to kinases. Hist-266 is conserved in Gβ2, Gβ3, and Gβ4, but is not in Gβ5, which has a lysine at the analogous position (43). Although we have no proof for this hypothesis at this time, we propose that, based on the high degree of homology mammalian, Gβ1Gβ4 can be phosphorylated at His-266. The mainly neuronally expressed Gβ5 functionally differs from the other Gβ subunits. In accordance with the lack of the respective histidine, we were not able to detect the phosphorylation of Gβ5γ2 (kindly provided by Dr. B. Nürnberg, Düsseldorf, Germany) in our reconstitution assay (data not shown). Two non-mammalian Gβ subunits are also lacking a histidine at the analogous position. These are Gβ2 of D. melanogaster, which has a proline at this position, and Gβ5 (Ste4p) from Saccharomyces cerevisiae. Interestingly, Ste4p has a 41-amino acid insertion at this position. Amino acids within this insertion are target to phosphorylation, which is necessary for adaptation of the mating response in yeast (44, 45). In summary, His-266 is apparently not required for forma-

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**Fig. 7. Localization of His-266 in the three-dimensional structure of the Go13-GDPβγ heterotrimer.** Ribbon diagram (A) and calotte model (B) of Go13-GDPγS (magenta), Gβ (blue), and Gγ (brown). Histidine residues are marked in white. An arrow points to the position of His-266. The GDP molecule is given in green.
tion of the seven propeller blade Gβ structure and is placed on the surface of the heterotrimer at a position where phosphorylation is likely to occur. Therefore, we propose that NDPK B phosphorylates the structurally exposed His-266 in Gβ in a stoichiometric complex with Gβγ.

A phosphotransfer from NTP to His-118 in NDPK B and subsequently onto His-266 of Gβ and further onto GDP might offer an explanation for several reports indicating a higher potency of NDPK-formed GTP compared with exogenously added GTP in G protein activation (10, 46, 47). However, the three-dimensional structure of the heterotrimeric G protein added GTP in G protein activation (10, 46, 47). However, the drastic increase in adenylyl cyclase activity as a consequence of a phosphotransfer from NTP to His-118 in NDPK B and subsequently onto His-266 of Gβ/H9252/H9253. Moreover, a low abundance of the NDPK-G protein complex of NDPK and G proteins do not co-purify and thus are not complexed with Gβγ. Nevertheless, the drastic increase in adenylyl cyclase activity as a result of an overexpression of NDPK B and G,α (42) might be an indication for the high efficiency of the phosphotransfer to activate G proteins.

Both purification procedures revealed that the vast majority of G proteins do not co-purify and thus are not complexed with NDPK B. Therefore, they are most likely not accessible to the phosphate transfer reaction. Although it is likely that purification, especially solubilization from the membrane environment, could cause a substantial dissociation of NDPK B from G proteins, the so far unknown scaffolding protein mentioned above could be the limiting factor within the complex of NDPK B and Gβγ. Moreover, a low abundance of the NDPK-G protein complex would fit into a concept where NDPK exclusively regulates the basal tone of G protein activities and where the vast majority of G proteins serve the "classical" receptor signal transduction, as discussed in the accompanying paper (42).

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