A novel regulatory mechanism for trimeric GTP-binding proteins in the membrane and secretory granule fractions of human and rodent β cells

Anjaneyulu KOWLURU,*†, Scott E. SEAVEY,* Christopher J. RHODES† and Stewart A. METZ*

*Department of Medicine and Section of Endocrinology, University of Wisconsin School of Medicine, Madison, WI, and William S. Middleton Memorial VA Hospital, 2500 Overlook Terrace, Madison, WI 53705, and †E.P. Joslin Research Laboratory, Joslin Diabetes Center, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02215, U.S.A.

Recently we described roles for heterotrimeric and low-molecular-mass GTP-binding proteins in insulin release from normal rat islets. During these studies, we observed that a protein with an apparent molecular mass (37 kDa) similar to that of the β subunit of trimeric GTP-binding proteins underwent phosphorylation in each of five classes of insulin-secreting cells. Incubation of the β cell total membrane fraction or the isolated secretory granule fraction (but not the cytosolic fraction) with [γ - 32 P]ATP or [γ - 32 P]GTP resulted in the phosphorylation of this protein, which was selectively immunoprecipitated by an antiserum directed against the common β subunit of trimeric G-proteins. Disruption of the $\alpha\beta\gamma$ trimer (by pretreatment with either fluoroaluminate or guanosine 5'-[γ -thio]triphosphate) prevented β subunit phosphorylation. Based on differential sensitivities to pH, heat and the histidine-selective reagent diethyl pyrocarbonate (and reversal of the latter by hydroxylamine), the phosphorylated amino acid was presumptively identified as histidine. Incubation of pure β subunit alone or in combination

with the exogenous purified α subunit of transducin did not result in the phosphorylation of the β subunit, but addition of the islet cell membrane fraction did support this event, suggesting that membrane localization (or a membrane-associated factor) is required for β subunit phosphorylation. Incubation of phosphorylated β subunit with $G_{\alpha\text{-GDP}}$ accelerated the dephosphorylation of the β subunit, accompanied by the formation of $G_{\alpha\text{-GTP}}$. Immunoblotting detected multiple α subunits (of G_i , G_o and G_q) and at least one β subunit in the secretory granule fraction of normal rat islets and insulinoma cells. These data describe a potential alternative mechanism for the activation of GTP-binding proteins in β cells which contrasts with the classical receptor-agonist mechanism: G_β undergoes transient phosphorylation at a histidine residue by a GTP-specific protein kinase; this phosphate, in turn, may be transferred via a classical Ping-Pong mechanism to $G_{\alpha\text{-GDP}}$ (inactive), yielding the active configuration $G_{\alpha\text{-GTP}}$ in secretory granules (a strategic location to modulate exocytosis).

INTRODUCTION

In most cells, transduction of extracellular signals involves ligand binding to a receptor, often followed by activation of one or more GTP-binding proteins (G-proteins) and their effector systems [1,2]. Pancreatic β cells are unusual in that the major physiological agonist, glucose, lacks an extracellular receptor. Instead, events consequent to glucose metabolism promote insulin secretion via the generation of diffusible second messengers, such as cations, cyclic nucleotides and lipid hydrolytic products generated by the action of phospholipases A_2 , D or C [3,4]. Nonetheless, glucose effects on insulin secretion from β cells seem to involve G-proteins. Several such proteins have been identified in various subcellular fractions (including an enrichment on secretory granules) of normal rat and human islets as well as insulin-secreting pure β (RIN or HIT) cells [5–9]. Blockade of the function of certain G-proteins by impeding their post-translational modifications selectively reduces fuel-induced insulin secretion [5,10]. Moreover, we have demonstrated a permissive role for GTP in nutrient-induced insulin secretion [11,12]. While the exact locus (loci) at which GTP exerts its regulatory role in insulin secretion has not been identified as yet, the possible targets may include one (or more) G-protein(s) [5–9]. Two major classes of such signal-transducing G-proteins have been identified in pancreatic β cells thus far (for a review, see [8]).

The first group is heterotrimeric in nature and consists of α (39–41 kDa), β (35–37 kDa) and γ (< 8 kDa) subunits; the second group is comprised of low-molecular-mass (ras-like; 18–27 kDa) G-proteins.

Typically, G-protein activation involves the conversion of the GDP-liganded (inactive) form to the GTP-bound (active) configuration; this is achieved by a direct exchange of GDP for GTP stimulated by receptor activation [1,2]. Alternatively, this step can be regulated by GTP/GDP exchange proteins, at least in the case of low-molecular-mass G-proteins [13]. A third potential mechanism may involve the direct phosphorylation of GDP (while it is still bound to G-proteins) to GTP, resulting in an active conformation. Such a reaction could be mediated by nucleoside diphosphokinase (NDP kinase), which catalyses the transfer of terminal phosphates from nucleotide triphosphates (i.e. ATP) to GDP, yielding GTP [14,15]. We have recently characterized NDP kinase activity in normal rat and human islets as well as in insulin-secreting pure β (RIN or HIT) cells [16]. There is emerging evidence in support of a direct phosphorylation of GDP bound to G-proteins [14,15]; however these observations have been met with some scepticism [17].

In the present study we describe a similar, but novel, potential activation mechanism for heterotrimeric G-proteins, which involves a transient phosphorylation of the β subunit, putatively at a histidine residue; this phosphate is then transferred to the

Abbreviations used: NDP kinase, nucleoside diphosphokinase; CK II, casein kinase II; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; GTP[S], guanosine 5'-[γ -thio]triphosphate; GTP[β S], guanosine 5'-[β -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; App[NH]p, adenosine 5'-[β -imido]triphosphate.

† To whom correspondence should be addressed at: Division of Endocrinology, H4/568 Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792, U.S.A.

GDP-bound (inactive) α subunits to yield GTP-bound (active) α subunits. Recently Wieland et al. [18] described a virtually identical phenomenon in HL-60 cell membranes. This mechanism may have a close analogy in bacterial sensory transduction systems [19,20]. Although the physiological role for this alternative mechanism remains to be defined, it could provide a receptor-independent link between fuel metabolism and the G-protein-linked exocytotic secretion of insulin.

Portions of these studies were presented at the 54th Annual Meeting of the American Diabetes Association, New Orleans, 1994 [20a], at the Annual Meeting of the American Federation for Clinical Research, Baltimore, 1994 [20b], and at the International Diabetes Federation Satellite Symposium on Pancreatic β Cell, Kyoto, Japan, November 1994.

MATERIALS AND METHODS

Materials

$[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (30 Ci/mmol), $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (600 Ci/mmol) and adenosine 5'- $[\gamma\text{-}^{35}\text{S}]\text{thio}[\text{triphosphate}]$ ($[\gamma\text{-}^{35}\text{S}]\text{ATP[S]}$) (1155 Ci/mmol) were purchased from NEN-Du Pont, Boston, MA, U.S.A. Nucleoside triphosphates and their non-hydrolysable analogues were purchased from Boehringer Mannheim, Indianapolis, IN, U.S.A. GDP, UDP, IDP, CDP, ADP, diethyl pyrocarbonate (DEPC), hydroxylamine, spermine, heparin [high-molecular mass (from intestinal mucosa), and low-molecular mass (6000 Da; prepared by enzymatic depolymerization)] and Protein A-agarose beads were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

A polyclonal antiserum directed against the 40–44 kDa α subunit of human casein kinase II (CK II) was obtained from Upstate Biotechnology, Inc., Lake Placid, NY, U.S.A. Antisera directed against various α subunits and the common β subunit of G-proteins were purchased from NEN-Du Pont. In some studies, an affinity-purified rabbit polyclonal antiserum raised against a synthetic 20-amino-acid peptide mapping within a highly conserved domain at the C-terminus of $G_{\beta 1}$ of mouse origin was employed. This antiserum and its peptide antigen were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A. Specificity characteristics of all the antisera employed in the present study are given in Table 1.

Purified subunits (α , β/γ) of transducin were generously provided by Dr. Akio Yamazaki, Kresge Eye Institute, Detroit, MI, U.S.A. HIT cells (passage 72) were provided by Dr. Paul Robertson and Dr. Hui-Jian Zhang, University of Minnesota Medical School, Minneapolis, MN, U.S.A. INS-1 cells (passages 65–76) were provided by Dr. Claes Wollheim, University of Geneva, Geneva, Switzerland. Human islets were provided by Dr. David Scharp, Washington University Medical School, St. Louis, MO, U.S.A.

Table 1 Specificity of different antisera used in the present study

Designation	G-protein subunit(s) identified	Specificity
AS/7	α	G_{α} , $G_{\beta 1}$, $G_{\beta 2}$
GO/1	α	$G_{\alpha} < G_{\beta 3}$
EC/2	α	$G_{\beta 3} < G_{\alpha}$
QL	α	G_{α} and $G_{\beta 11}$
SW/1	β	Common β
T-20	β	Common β

Isolation of total membrane and secretory granule fractions from insulin-secreting cells

Pancreatic islets were isolated from Sprague–Dawley rats (300–400 g body weight) by a collagenase digestion method, as described in [5,6,9,11,12]. Islets were manually picked twice under stereomicroscopic control to avoid any contamination by exocrine acinar tissue. Secretory granules from rat pancreatic islets were isolated by the differential centrifugation method as described in [6,9]. Details of the purity of this fraction determined by marker enzymes such as $\text{Na}^+/\text{K}^+\text{-ATPase}$ (plasma membrane), acid phosphatase (lysosomes), succinic isodonitrotetrazolium-reductase (mitochondria), UDP galactosyltransferase (Golgi), lactate dehydrogenase (cytosol) and insulin (secretory granules), as well as by electron microscopy, have been described in detail elsewhere [6,9]. Rat insulinoma tissue was propagated in New England Deaconess Hospital (NEDH) rats as previously described [21]. A purified insulin secretory granule fraction was prepared from this rat insulinoma by differential, Nycodenz (Nyegaard & Co., Oslo, Norway) and Percoll (Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.) gradient centrifugation, and was then characterized by marker enzyme analysis as previously described [22]. Membrane fractions from normal rat islets, normal human islets and HIT cells were isolated by a differential centrifugation method as described in [9].

Phosphorylation of the β subunit and its quantification

The phosphorylation reaction was carried out (total volume of 100 μl) in 50 mM Tris/HCl, pH 7.4, 2 mM dithiothreitol (DTT), 3 mM MgCl_2 (or other metal ions, as indicated), secretory granule protein (5–10 μg) or total membrane fraction (20–30 μg of protein) and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ or $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1–2 $\mu\text{Ci}/\text{tube}$; 8–12 μM) at 37 °C for 2 min. The phosphorylation reaction was linear up to 5 min under these experimental conditions. The reaction was terminated by the addition of Laemmli stop solution [5,9]. Since the phosphohistidine of the β subunit is heat-labile (see below), samples were incubated in the sample buffer at room temperature for 30 min prior to SDS/PAGE. Following separation on SDS/12%–PAGE, gels were routinely fixed in methanol/acetic acid/water (4:1:5; by vol.) for 1.5 h and dried at room temperature. Short fixation times were chosen in order to prevent the loss of label from phosphohistidine under acidic pH conditions [16,18]. Labelled proteins were identified by autoradiography, and their labelling intensity was quantified by scanning individual lanes using a Zeineh Video Laser Densitometer (Biomed Instruments, Inc., Fullerton, CA, U.S.A.) that was interfaced to an IBM computer equipped with software to calculate the individual peak areas [9].

Immunoprecipitation of phosphorylated β subunit

Immunoprecipitation of the β subunit was carried out according to Wieland et al. [18], with minor modifications. In brief, labelled secretory granule or membrane proteins were solubilized in a buffer consisting of 25 mM EDTA, pH 7.4, 0.8% SDS and 0.2 μM PMSF. Following solubilization at 4 °C for 20 min, labelled proteins were incubated with an antiserum directed against the C-terminus of β subunit common to trimeric G-proteins (typically, at a 1:750–1000 dilution) or preimmune serum in a precipitation buffer consisting of 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 25 mM EDTA, 1 mM DTT, 1 mM NaF, 1% sodium deoxycholate, 0.3 μM aprotinin, 0.2 μM PMSF, 10 μM guanosine 5'- $[\gamma\text{-thio}]\text{triphosphate}$ (GTP[S]) and 1% Nonidet P-40. For antigen blocking (i.e. competition) experiments, anti- G_{β} serum (2 μg of protein) was preincubated with

20 μ g of control peptide (raised against a synthetic 20-amino-acid peptide mapping within a highly conserved domain at the C-terminus of the β_1 subunit of mouse origin) in 30 mM sodium phosphate buffer, pH 7.4, containing 125 mM NaCl, 0.1 % sodium azide and 0.03 % gelatin at 25 °C for 2 h before being used for immunoprecipitation. Immune complexes were sedimented using Protein A-agarose beads (16 mg/ml), and pellets were washed extensively ($\times 3$) with 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl. Next, 75 μ l of SDS/PAGE sample buffer was added to the immunoprecipitates, which were then incubated at room temperature (to prevent heat-associated loss of radioactivity from phosphohistidines; see below) for 30 min prior to SDS/PAGE. Proteins in the sample buffer eluates were subjected to SDS/PAGE (12 %). Following this, gels were processed for autoradiography as described above.

pH and temperature stability of the phosphorylated β subunit

To determine the stability of the phosphoproteins in acidic and alkaline pH conditions, labelled secretory granule or membrane proteins were incubated in water (as control), 1 M HCl or 1 M NaOH for 30 min at 25 °C [16,18]. Samples were then separated by SDS/PAGE; the degree of residual labelling was determined by autoradiography. In order to determine the temperature stability of phosphorylated amino acids, samples were incubated either at 25 °C for 30 min (as above) or at 95 °C for 10 min [16]. Following these incubations, proteins were separated by SDS/PAGE and the degree of residual 32 P labelling was quantified by autoradiography.

Sensitivity of β subunit phosphorylation to DEPC and its reversal by hydroxylamine

DEPC reacts with histidine residues in proteins to yield an N-carboxyhistidine derivative [23,24]. It has been suggested that DEPC is selective for histidine residues in proteins at pH 6.0, since most nucleophiles exhibit greater reactivity in their unprotonated forms [25]. Therefore DEPC pretreatment (0–10 mM) of β cell membrane proteins was carried out in sodium phosphate buffer, pH 6.0–6.2 (total volume of 150 μ l), for 10 min at room temperature. Following the reaction, 1 ml of ice-cold 50 mM Tris/HCl, pH 7.4, and membranes were isolated by centrifugation and were reconstituted in homogenization buffer. β subunit phosphorylation was carried out in these membrane fractions as described above.

In studies that addressed the reversal by hydroxylamine of DEPC-induced inhibition of β subunit phosphorylation, DEPC-treated membranes (see above) were incubated in 0.1 M acetate buffer, pH 5.1, containing 0.6 mM NaCl in the presence or absence of hydroxylamine (0.55 M), as described in [26]. Hydroxylamine was freshly prepared by mixing 2 vol. of 8 M NaOH with 5 vol. of 4 M hydroxylamine at 4 °C [26]. Incubations were carried out at 25 °C for 20 min. Control membranes were incubated with diluents under identical conditions. Following incubation, membranes were washed by centrifugation and β subunit phosphorylation was examined in these membranes as described above.

Dephosphorylation of β subunit in the presence of GDP or $G_{\alpha-GDP}$

Total membrane or secretory granule proteins were prelabelled (in a total volume of 100 μ l) with [γ - 32 P]ATP for 2 min (as described above), and the reaction was terminated by adding 400 μ l of ice-cold buffer consisting of 10 mM Tris/HCl, pH 7.4, and 5 mM EDTA. The contents of the tubes were then centrifuged at 30 000 g for 10 min at 4 °C. The pellets were washed once more

(as above) to remove any unreacted label and were reconstituted in a medium consisting of 50 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and 150 mM NaCl. Dephosphorylation of the β subunit was carried out at 37 °C for different time intervals, as described in the Results section, in a medium consisting of 50 mM Tris/HCl, pH 7.4, 3 mM $MgCl_2$ and 1 mM DTT in the presence of diluent alone, GDP (0–100 μ M) or $G_{\alpha-GDP}$ (prepared by incubating pure α subunit of transducin with 1 μ M GDP at 4 °C for 24 h [27]). The reaction was terminated by adding Laemmli stop solution. Proteins were separated by SDS/PAGE and the residual labelling of the β subunit was determined by autoradiography.

Formation of $G_{\alpha-GTP}$ from $G_{\alpha-GDP}$ via phospho-relay

Total membrane fraction from normal rat islets (250–300 μ g of protein) was prelabelled with either [γ - 32 P]ATP (as above) for 3 min or with [γ - 35 S]ATP[S] (1115 Ci/mmol; 300 μ Ci/ml) for 20 min at 37 °C, as described above. The reaction was terminated by adding 5 vol. of ice-cold homogenization buffer, and labelled membranes were washed by centrifugation at 105 000 g for 30 min and reconstituted in homogenization buffer for phospho-relay assays. In these assays, membranes that had been prelabelled with [γ - 32 P]ATP were incubated for 15 min at 25 °C (in a total volume of 100 μ l) in the presence or absence of transducin α subunit liganded to guanosine 5'-[β -thio]diphosphate (GDP[S]). Likewise, membranes that were labelled with [γ - 35 S]ATP[S] were incubated in the presence or absence of transducin α subunit liganded to GDP. The reactions were terminated by adding ice-cold buffer consisting of 30 mM EDTA, pH 7.4, and 1 mM GTP[S]. Bound nucleotides were extracted according to a procedure described by Draznin et al. [28] using an extraction medium consisting of 1 M KH_2PO_4 , 2 mM EDTA, 2 mM DTT, 0.2 % SDS and 500 μ M each of GTP and GDP. Samples were heated at 85–90 °C for 3 min and applied to polyethyleneimine-cellulose plates (E. M. Separations, Gibbstown, NJ, U.S.A.) for the separation of GTP[S] and guanosine 5'-[β -thio]triphosphate (GTP[β S]) as described in [6,16]. Radioactivity associated with nucleotide triphosphates was quantified by scintillation spectrometry.

Identification by immunoblotting of subunits of heterotrimeric G-proteins in the secretory granule fraction

Secretory granule proteins were separated by SDS/PAGE (12 %) and transferred on to a nitrocellulose membrane using a Bio-Rad transblot apparatus [5,9]. Non-specific protein binding sites were blocked using gelatin. The membranes were then incubated with various antisera or preimmune serum (Table 1; 1:1000 dilution) for 15 h at room temperature with gentle shaking. Immune complexes were identified by colour development using alkaline phosphatase coupled to anti-rabbit IgG (Bio-Rad, Hercules, CA, U.S.A.). Molecular masses of proteins were determined using kaleidoscope pre-stained standards from Bio-Rad.

Other methods

Protein concentrations in β cell subcellular fractions were assayed according to Bradford using BSA as a standard [29].

RESULTS

Phosphorylation of G-protein β subunit in the total membrane and secretory granule fractions of insulin-secreting cells

A 37 kDa protein was consistently phosphorylated (in the presence of either [γ - 32 P]ATP or [γ - 32 P]GTP) in the total par-

Figure 1 Phosphorylation of a 36–37 kDa protein in the membrane fractions of normal rat islets, human islets and HIT cells

The phosphorylation reaction was carried out for 2 min (as described in the Materials and methods section) using total particulate fractions from normal rat islets (lane A), human islets (lane B) or HIT cells (lane C), with [γ - 32 P]ATP as phosphoryl donor. Labelled proteins were separated by SDS/PAGE and identified by autoradiography. A 37 kDa protein (indicated by the arrow) was consistently labelled in all three insulin-secreting cells studied. Data are representative of seven experiments using rat islets and two experiments each using human islets and HIT cells.

Figure 2 Bivalent-cation-dependent phosphorylation of the 37 kDa protein in the secretory granule fraction of rat insulinoma cells

Phosphorylation of insulinoma cell secretory granule proteins was carried out for 2 min using [γ - 32 P]ATP as phosphoryl donor, in the presence of 3 mM Mg^{2+} or Mn^{2+} . Labelled proteins were identified by autoradiography following SDS/PAGE. No significant labelling of these proteins was observed in the absence of bivalent metal ions (i.e. in the presence of 3 mM EDTA; results not shown). Data are representative of at least five experiments with similar results using Mg^{2+} and two experiments using Mn^{2+} .

ticulate fraction of normal rat islets within 2 min of incubation at 33 °C [16,30]. Phosphorylation of this protein was linear up to 5 min under these conditions (additional results not shown). In studies using [γ - 32 P]ATP as phosphoryl donor, we observed (Figure 1) that at least three to seven proteins, including a 37 kDa protein (indicated by the arrow), were phosphorylated in normal rat islet (lane A), normal human islet (lane B), HIT cell (lane C) and INS-1 cell (results not shown) membranes. Since we have found that heterotrimeric G-proteins associate with islet

Figure 3 Selective GTP-dependent phosphorylation of the 37 kDa protein in a rat islet secretory granule fraction

Rat islet secretory granule proteins were phosphorylated for 2 min in the presence of [γ - 32 P]GTP as phosphoryl donor and 3 mM Mg^{2+} . To saturate ATP binding sites, ATP[S] and App[NH]p (1 mM each) were also included in the assay mixture where indicated (+). At least four proteins were labelled in the absence of ATP analogues (as indicated by arrows), including a 37 kDa protein (indicated by the bold arrow). In the presence of ATP analogues only the 37 kDa protein was labelled. Occasionally we also observed phosphorylation of a protein in the region 100–120 kDa (indicated by the broken arrow). Data are representative of seven to ten experiments with identical results.

secretory granules ([6,8,9]; see the Discussion section), we extended these observations to the secretory granule fraction. Using secretory granules isolated from insulinoma cells, at least seven proteins, including a 37 kDa protein (indicated by the solid arrow), were phosphorylated using [γ - 32 P]ATP as phosphoryl donor in the presence of bivalent cations (3 mM Mg^{2+} or Mn^{2+} ; Figure 2). The labelling of the 37 kDa protein was comparable in the presence of either of these ions (Figure 2). Calcium (3 mM) was much less effective (< 20%) than Mg^{2+} or Mn^{2+} (additional results not shown). Similar metal ion requirements for the phosphorylation of the 37 kDa protein were observed using rat islet secretory granule and membrane fractions (results not shown). When using [γ - 32 P]GTP as phosphoryl donor at least four proteins, including the 37 kDa protein, were labelled in the rat islet secretory granule fraction within 2 min of incubation (indicated by the bold arrow in Figure 3). Additionally, inclusion of ATP[S] and adenosine 5'-[β - γ -imido]triphosphate (App[NH]p) (1 mM each) in the phosphorylation mixture largely abolished the labelling of all proteins except the 37 kDa protein after this short period of incubation (Figure 3). Under these conditions, ATP[S] (1 mM) alone even increased the phosphorylation of the 37 kDa protein (+36%; $n = 2$), compatible with the recent observations of Wieland et al. [18]. The observed inhibition of phosphorylation of these other proteins by non-hydrolysable ATP analogues, even during short incubation periods (2 min), may be due to saturation of non-specific NTP binding sites on their putative kinases as well as inhibition of GTP-degrading nucleotidase activity [18]. On occasions, a protein of approx. 100–120 kDa (indicated by the broken arrow in Figure 3) was also labelled, even in the presence of ATP[S] and App[NH]p. Using [γ - 35 S]ATP[S] or [γ - 35 S]GTP[S], thiophosphorylation of the 37 kDa protein was observed, albeit at a much slower rate. For example, using [γ - 35 S]ATP[S] or GTP[S], thiophosphorylation of the 37 kDa protein was demonstrable only after 20 min of incubation, in contrast to a rapid (linear up to only 5 min) phosphorylation when ATP or GTP were used as phosphoryl

Figure 4 Immunoprecipitation of the 37 kDa phosphoprotein from rat insulinoma secretory granule (a) and rat islet membrane (b) fractions by an antiserum directed against the common β subunit of heterotrimeric G-proteins

(a) Rat insulinoma secretory granule proteins were labelled in the presence of [γ - 32 P]GTP as a phosphoryl donor but in the absence of ATP[S] and App[NH]p as indicated in the text. Using an insulinoma secretory granule fraction, several proteins were phosphorylated under these assay conditions. SDS-solubilized labelled membrane proteins were incubated with an antiserum directed against the common β subunit (+) and immune complexes were sedimented using Protein A conjugated to agarose (see the Materials and methods section). In order to prevent the heat-associated loss of the labelling of phosphohistidine, proteins in the immunoprecipitates were incubated with sample buffer at room temperature for 30 min prior to SDS/PAGE. Labelled proteins were identified by autoradiography. Data are representative of two experiments having similar results. (b) Membrane proteins were prelabelled with [γ - 32 P]GTP in the absence of ATP[S] and App[NH]p (lane B) and immunoprecipitated using an antiserum directed against the β subunit common to trimeric G-proteins (lane A). To determine the specificity of this immunoprecipitation, β subunit antiserum was incubated with a 10-fold (w/w) excess of antigen peptide (see the Materials and methods section for details) prior to use for immunoprecipitation (lane C). These data indicate that neutralization of the antiserum with control antigen peptide completely blocked the immunoprecipitation of the labelled β subunit.

donors (additional results not shown). These data suggest that the putative kinase preferentially uses ATP or GTP over their thio analogues (see below). Since the labelling of the 37 kDa protein was more selective using GTP, we used [γ - 32 P]GTP as substrate in the presence of App[NH]p and ATP[S] (1 mM) in most of the studies described below, unless stated otherwise.

Immunoprecipitation of the 37 kDa phosphoprotein using antiserum directed against the β subunit of heterotrimeric G-proteins

Rat islet total membrane or insulinoma cell secretory granule proteins were phosphorylated in the presence of [γ - 32 P]GTP. In these studies, the phosphorylation reaction was carried out in the absence of ATP[S] and App[NH]p; under these conditions of assay, using insulinoma secretory granules, several proteins were phosphorylated, compatible with data presented in Figure 2. However, data in Figures 4(a) and 4(b) indicate that, of these labelled proteins, the 37 kDa phosphoprotein was selectively immunoprecipitated from the secretory granule (Figure 4a) and membrane (Figure 4b) fractions by the antiserum to the common β subunit. Preincubation of β subunit antiserum with peptide antigen (see the Materials and methods section for details) completely blocked the immunoprecipitation of the labelled β subunit from the membrane fraction (Figure 4b). Labelled β subunit could not be precipitated using preimmune serum (results not shown). In these experiments, however, a significant

Table 2 A membrane-associated factor is required for β subunit phosphorylation

An islet membrane fraction (10–15 μ g of protein) was incubated with [γ - 32 P]ATP in the absence or presence of purified α and β subunits of transducin (see the Materials and methods section). Labelled proteins were separated by SDS/PAGE and identified by autoradiography. The intensity of the phosphorylated β subunit was quantified by densitometry. Data are means $\pm$ S.E.M. from the number of determinations indicated in parentheses.

Additions	β subunit phosphorylation (arbitrary units)
Islet membrane alone	242 $\pm$ 42 (3)
β subunit alone	22 (2)
β subunit plus α subunit	9 (2)
Islet membrane plus β subunit	670 $\pm$ 20 (3)

loss and/or incomplete recovery of β -subunit-associated radioactivity was observed during the immunoprecipitation procedure. This phenomenon has been previously demonstrated in the case of NDP kinase, another protein phosphorylated at a histidine residue [16,31]. Recovery experiments using purified transducin β subunit which had been phosphorylated in the presence of an islet membrane fraction indicated that only 15–20 % of the labelling in the β subunit was recoverable under these experimental conditions. Such modest recoveries are most likely due to the fact that we did not employ conventional heat treatment to dissociate the immune complexes from the Protein A-agarose conjugate, since heat treatment results in loss of label from phosphohistidine ([16,18]; see below). Indeed, under these experimental conditions, we observed that significant amounts (70 %) of the labelling associated with the β subunit in the immunoprecipitates remained associated with agarose beads after elution with SDS-sample buffer (1134 $\pm$ 48 c.p.m. in SDS-sample buffer eluates versus 2860 $\pm$ 324 c.p.m. remaining in agarose beads; n = 6 each). Nonetheless, these data indicate that the phosphorylated 37 kDa phosphoprotein does contain the β subunit of heterotrimeric G-proteins.

A membrane-associated factor is required for phosphorylation of the β subunit

Immunological identification of the phosphorylated 36–37 kDa protein as the β subunit of trimeric G-protein(s) prompted us to examine whether the purified β subunit of transducin is phosphorylated by islet fractions. Data in Table 2 indicate that incubation of the β subunit, alone or in combination with the purified α subunit of transducin, did not result in the phosphorylation of the β subunit. However, incubation of the β subunit with the islet membrane fraction (Table 2), but not with islet cytosol (results not shown), resulted in significant phosphorylation of the β subunit. As indicated in Figure 5, concentration-dependent (Spearman's r = 0.943; P < 0.005) and saturable phosphorylation of the β subunit was demonstrable in the presence of the islet membrane fraction. These data indicate that the phosphorylation of the β subunit may require a membrane-associated factor (or its insertion in the membrane).

Putative identification of the phosphoamino acid of the β subunit

Since different phosphoamino acids have different and characteristic patterns of stability at acidic and alkaline pH or at different temperatures [16,18], we examined the stability of the phosphoamino acid of the β subunit. The labelling of the β

Figure 5 Phosphorylation of purified transducin β subunit in the presence of islet membranes

Islet membranes (10–15 μ g of protein) were incubated for 2 min in the presence of increasing concentrations of purified β subunit of transducin and [γ - 32 P]ATP, as indicated in the text. Labelled proteins were separated by SDS/PAGE and identified by autoradiography. The intensity of labelling was quantified by densitometry. The degree of phosphorylation at each concentration of β subunit is plotted.

Figure 6 Stability characteristics of the phosphoamino acid(s) of the phosphorylated β subunit in a rat islet secretory granule fraction: putative identification as phosphohistidine

Rat islet secretory granule proteins were phosphorylated for 2 min in the presence of [γ - 32 P]GTP and Mg^{2+} (3 mM) as described in the text, and the reaction was terminated by adding 10 mM EDTA to the assay mixture. In experiments addressing the stability of the phosphorylated β subunit, labelled proteins were incubated in the presence of water (lane A), 1 M HCl (lane B) or 1 M NaOH (lane C) for 30 min at room temperature. Following this, samples were neutralized with either NaOH or HCl and subjected to SDS/PAGE. To examine the heat-stability of the label in the phosphorylated β subunit, samples were heated at 95 °C for 10 min and subjected to SDS/PAGE (lane D). Labelled proteins were then identified by autoradiography. Data are representative of two experiments demonstrating similar stability characteristics.

subunit in the secretory granule fraction was significantly decreased (> 80 %) under acidic pH (1 M HCl) conditions (Figure 6); a more modest (< 30 %) loss of labelling of the β subunit after exposure to alkaline pH (1 M NaOH) was also observed. Heat treatment (95 °C for 10 min) completely abolished the labelling of the β subunit. Similar differential sensitivities of phosphorylated β subunit to heating were demonstrable in the membrane fractions from normal rat islets, HIT cells or INS-1 cells (results not shown).

Since these data suggested that a majority of the labelling of the β subunit might be via a phosphoramidate linkage at a histidine residue [16,18], they were extended by the use of DEPC, which reacts selectively with histidine residues to yield an *N*-carbethoxyhistidine derivative [23,24] and to block accessibility to phosphate transfer. Exposure of a membrane fraction from normal rat islets to DEPC largely abolished the [γ - 32 P]GTP-dependent phosphorylation of the β subunit (Figure 7). This could be observed at a concentration of DEPC at least as low as 2.5 mM. A similar inhibitory effect of DEPC on β subunit

Figure 7 Inhibition by DEPC of β subunit phosphorylation in islet membranes and its reversal by hydroxylamine

Islet membranes were preincubated in the absence or presence of DEPC (2.5 mM) for 10 min at 25 °C. Following incubation, membranes were washed by centrifugation and reincubated for 20 min at 25 °C with either diluent or freshly prepared hydroxylamine (HA; 0.55 M) as indicated. Following washing, membrane proteins were then phosphorylated using [32 P]GTP as phosphoryl donor in the presence of ATP[S] and App[NH]p as described in the text. Proteins were separated by SDS/PAGE and processed for autoradiography. Shown here is a representative autoradiograph from two independent experiments having identical results.

Figure 8 Effect of DEPC on phosphorylation of HIT cell proteins

HIT cell homogenates were preincubated with increasing concentrations (0–10 mM) of DEPC at room temperature for 10 min prior to the phosphorylation assay (see the Materials and methods section). Proteins were labelled with [γ - 32 P]ATP as described in the text. Labelled proteins were separated by SDS/PAGE and identified by autoradiography. The phosphorylated β subunit is indicated by an arrow. Shown here is an autoradiograph representative of five experiments with comparable results.

phosphorylation was also demonstrable in the secretory granule fraction (results not shown). Furthermore, treatment of DEPC-exposed membranes with hydroxylamine (see the Materials and methods section), an agent which can reverse histidine ethoxycarbonylation [18,26], completely restored β subunit phosphorylation (Figure 7). Moreover, labelling of the β subunit was inhibited (> 90 %; additional results not shown) by direct exposure to hydroxylamine, which is known to hydrolyse high-energy phosphates from acylphosphates and phosphoramidates [18,23–26]. In order to rule out non-specific inhibitory effects of DEPC on protein phosphorylation, the latter was studied using HIT cell homogenates and [γ - 32 P]ATP as phosphoryl donor in the absence or presence of increasing concentrations of DEPC (0–10 mM). The data in Figure 8 indicate that DEPC inhibited the phosphorylation of the β subunit in a concentration-dependent manner. More importantly, these data also indicate that this inhibition by DEPC was selective for the β subunit. Inhibition of β subunit phosphorylation was demonstrable at

Figure 9 A heterotrimeric conformation of G-proteins is required for the phosphorylation of the β subunit

(a) Rat insulinoma secretory granules were preincubated at room temperature for 10 min with GTP[S] (1 μ M) or for 30 min with fluoroaluminate (50 μ M) and then were phosphorylated in the presence of [γ -³²P]GTP and ATP[S] plus App[NH]p (1 mM each). Labelled proteins were identified by autoradiography. Similar data were obtained in another independent experiment using rat islet secretory granule proteins. (b) Rat islet membrane proteins were preincubated on ice for 10 min with increasing concentrations of GTP[S] (0–1 μ M) and then phosphorylated using [γ -³²P]ATP as described in the Materials and methods section. Labelled proteins were separated by SDS/PAGE and identified by autoradiography. The phosphorylated β subunit is indicated by an arrow. The degree of phosphorylation of the β subunit was quantified by densitometry.

DEPC concentrations as low as 0.5 mM. Phosphorylation of other proteins either was unaffected or (for proteins in the molecular mass range 45–60 kDa) was even stimulated by DEPC, especially at those concentrations (i.e. 5–10 mM) at which it clearly inhibited β subunit phosphorylation. Together, these data (lability at acidic pH, resistance to alkaline pH, lability to heat treatment, sensitivity to DEPC and its reversal by hydroxylamine) strongly indicate that the β subunit is phosphorylated via a phosphoramidate linkage, presumably at a histidine residue.

A trimeric conformation may be required for phosphorylation of the β subunit

In order to define whether the trimeric conformation (i.e. complexation of the $\alpha\beta\gamma$ subunits) is required for β subunit phosphorylation, secretory granule proteins were preincubated with GTP[S] (1 μ M) or fluoroaluminate (50 μ M); both of these agents dissociate the α subunit from the β/γ subunit complex of trimeric G-proteins [1,2]. Pretreatment with GTP[S] inhibited (> 90 %) the subsequent [γ -³²P]GTP-dependent phosphorylation of the β subunit in the secretory granule fraction (Figure 9a). Further studies indicated that GTP[S] inhibited β subunit phosphorylation even at concentrations as low as 10 nM (additional results not shown). These data suggest that GTP[S] pretreatment interferes with β subunit phosphorylation by dis-

Figure 10 Time course of dephosphorylation of the β subunit: acceleration in the presence of GDP or $G_{\alpha-GDP}$

Rat islet membranes were prelabelled in the presence of [γ -³²P]ATP as described in the text. Following labelling, membranes were washed extensively to remove the unreacted label and were then incubated at 37 °C for different time intervals (0–20 min) with diluent alone (○), GDP alone (▼) or the α subunit of transducin liganded to GDP (▽). Residual labelling of the β subunit was quantified by densitometry of the autoradiograms of SDS/PAGE gels (12%).

sociating the α subunit from the $\beta\gamma$ complex. We cannot totally exclude the possibility of GTP[S]-mediated thiophosphorylation of the β subunit at the histidine residue (see above and [32,33]). However, this seems unlikely, especially if one considers the rapidity with which GTP or GTP[S] binds to the G-protein α subunit, leading to the dissociation of $G_{\alpha-GTP}$ and the $\beta\gamma$ complex ([8] and references therein), in contrast to the slow time course of thiophosphorylation of the β subunit (see above). Furthermore, in order to minimize the kinase-mediated GTP[S]-dependent thiophosphorylation of the β subunit, a rat islet membrane fraction was incubated with increasing concentrations of GTP[S] (0–1 μ M) on ice for 10 min prior to studying β subunit phosphorylation. Under these conditions, GTP[S] can still bind to the α subunit(s) of trimeric G-protein(s) and dissociate the $\beta\gamma$ complex [27]. We then quantified phosphorylation of the β subunit in these preparations using [γ -³²P]ATP as substrate. Data in Figure 9(b) clearly indicate a marked inhibition of β subunit phosphorylation in membrane preparations incubated with GTP[S] (> 85 % inhibition in the presence of < 100 nM GTP[S]; Figure 9b). These data strongly suggest that GTP[S] inhibits β subunit phosphorylation by dissociating $\alpha\beta\gamma$ trimeric conformation. Furthermore, as a second approach, we used fluoroaluminate to dissociate the trimeric conformation. Fluoroaluminate pretreatment also reduced (–55 %) β subunit labelling (Figure 9a), further suggesting that an intact trimeric conformation is required for phosphorylation of the β subunit.

Phospho-relay from the β subunit to GDP or $G_{\alpha-GDP}$

An examination of the time course of dephosphorylation of the β subunit (Figure 10) indicated that > 50 % of the label was lost within 20 min of incubation. However, inclusion of either GDP (10 μ M) or $G_{\alpha-GDP}$ resulted in a marked acceleration in the dephosphorylation of the β subunit (Figure 10). More than 40–50 % of the label was lost within 10 min of incubation in the

Figure 11 Dephosphorylation of the β subunit by free GDP or $G_{\alpha}\text{-GDP}$ in secretory granule fractions

Rat islet secretory granule proteins were prelabelled in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as phosphoryl donor. Dephosphorylation of the β subunit was monitored (see the Materials and methods section) in the presence of diluent alone (—), GDP (1 μM ; **a**; +) or $G_{\alpha}\text{-GDP}$ (**b**; +) for 10 min at 37 °C. Residual labelling of the β subunit was determined by autoradiography following SDS/PAGE. Data are representative of two experiments (one using rat islet secretory granules and the other employing insulinoma granules).

Figure 12 Dephosphorylation of the β subunit in rat islet membranes: NDP specificity

A rat islet membrane fraction was prelabelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the text. Following this, membranes were washed by centrifugation and incubated in the presence of diluent or various NDPs (100 μM) for 10 min at 37 °C. Proteins were separated by SDS/PAGE (12%) and residual labelling of the β subunit (indicated by the arrow) was determined by autoradiography. Data are representative of two experiments with identical results.

presence of GDP or $G_{\alpha}\text{-GDP}$. These observations were then extended to the secretory granule fraction. ^{32}P -labelled secretory granule proteins were incubated with either free GDP (10 μM) or purified α subunit of transducin liganded to GDP (1 μM), and loss of labelling from the 37 kDa protein was measured. A significant decrease in the labelling of the β subunit was observed in the presence of either free GDP (Figure 11a) or GDP liganded to the α subunit (Figure 11b). As a negative control, GDP-liganded α subunit was heat-inactivated (95 °C for 10 min) and used for phospho-relay studies. Under these conditions, we failed to detect any loss of labelling from the β subunit (results not shown). These data may suggest that the native conformation of the α subunit (bound to GDP) is required for it to act as a 'phosphate-receiver' molecule. Specificity experiments using the total membrane fraction from normal rat islets indicated that, of all NDPs tested (i.e. ADP, GDP, IDP, CDP and UDP), only GDP and to a lesser degree IDP were effective in serving as receivers of phosphate from the β subunit (Figure 12). A similar NDP specificity has been demonstrated by Wieland et al. [18] for dephosphorylation of the β subunit from HL-60 membranes. These data (Figures 10–12) suggest that a specific phospho-relay

Table 3 Emergence of $G_{\alpha}\text{-GTP}$ from $G_{\alpha}\text{-GDP}$ and phosphorylated β subunit

Islet membranes were prelabelled with either $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (approach 1) or $[\gamma\text{-}^{35}\text{S}]\text{ATP}[\text{S}]$ (approach 2) as described in the Materials and methods section. After extensive washing to remove unreacted label, membranes were incubated with purified α subunit of transducin liganded to GDP[S] (approach 1) or GDP (approach 2) to facilitate transfer of phosphate groups from the β subunit to yield $\text{GT}^{32}\text{P}[\beta\text{S}]$ or GTP^{35}S . NTPs were extracted (see the Materials and methods section) and separated by polyethyleneimine TLC as described in [6,16]. Data are means $\pm$ S.E.M. from three independent measurements. Significance of differences from control: * $P = 0.0001$; ** $P = 0.0024$.

Approach 1	$G_{\alpha}\text{-GTP}[\beta\text{S}]$ formed (c.p.m.)
Membrane alone	5481 $\pm$ 323
Membrane + exogenous $G_{\alpha}\text{-GDP}[\text{S}]$	12178 $\pm$ 317*
Approach 2	$G_{\alpha}\text{-GTP}[\text{S}]$ formed (c.p.m.)
Membrane alone	189 $\pm$ 7
Membrane + exogenous $G_{\alpha}\text{-GDP}$	354 $\pm$ 23**

might occur from the β subunit to either free GDP or GDP liganded to the α subunit.

The emergence of $G_{\alpha}\text{-GTP}$ from $G_{\alpha}\text{-GDP}$ and the phosphorylated β subunit

We next determined whether the phosphate from the β subunit can be transferred to GDP bound to the α subunit via a Ping-Pong mechanism analogous to that used by NDP kinase [15,16]. In order to demonstrate the actual phospho-relay from the β subunit to the GDP bound to G_{α} , we developed two different experimental approaches. These were based on the quantification of the formation of analogues of GTP which are resistant to hydrolysis by the GTPase activities intrinsic to endogenous islet G-proteins [9]. In the first approach, the β subunit was prelabelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and transfer of this phosphate to GDP[S] liganded to the G_{α} subunit to yield $\text{GT}^{32}\text{P}[\beta\text{S}]$ was monitored. In the second approach, the β subunit was prelabelled with $[\gamma\text{-}^{35}\text{S}]\text{ATP}[\text{S}]$, and transfer of this sulphate to GDP liganded to the G_{α} subunit to yield GTP^{35}S was quantified. The data in Table 3 indicate that a greater than 2-fold increase in the formation of NTPs [i.e. $\text{GT}^{32}\text{P}[\beta\text{S}]$ (approach 1) or GTP^{35}S (approach 2)] was observed when labelled membranes were incubated with α subunits liganded to NDPs. These data indicate the emergence of a GTP-bound (active) form of G_{α} upon incubation of phosphorylated β subunit with $G_{\alpha}\text{-GDP}$ (see the Discussion section).

Inhibition of β subunit phosphorylation by UDP

We have recently demonstrated in insulin-secreting cells that UDP inhibits ($K_i = 2$ mM) the activity of NDP kinase (which catalyses the formation of GTP from ATP and GDP), probably by forming an abortive complex with the enzyme [15,16]. Some NDP kinase activity was associated with secretory granule fractions [16]. Since NDP kinase is also phosphorylated on a histidine residue [16], we examined the effects of UDP on the phosphorylation of the β subunit. UDP (10 mM) almost completely abolished (> 90 %) the labelling of the β subunit using $[\gamma\text{-}$

Figure 13 The pure $\beta\gamma$ subunit of trimeric G-proteins lacks transphosphorylating activity using free NDPs as substrates, in contrast to NDP kinase

The transphosphorylating activity of pure NDP kinase (NDPK; from baker's yeast; 2 μ g) and pure β subunit (transducin; 5 μ g) was determined by monitoring their ability to catalyse the formation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and GDP) or $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (in the presence of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and ADP). Labelled NTPs were separated by polyethyleneimine TLC using 0.75 M KH_2PO_4 , pH 3.65, as described in [6,16].

$^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ as substrate, suggesting that a phospho-transfer mechanism similar to that used by NDP kinase [15,16,30,31], or NDP kinase itself, may be involved in β subunit phosphorylation.

Preliminary characterization of the kinase catalysing the phosphorylation of the β subunit

The above data (Figures 2, 3 and 6) indicate close similarities between NDP kinase and the β subunit, namely nucleotide specificity and metal ion requirements for phosphorylation at a histidine residue, and inhibition by UDP [16]. Therefore we determined whether the β subunit undergoes auto-phosphorylation (as does NDP kinase), and whether it is capable of mediating the transphosphorylation of free NDPs to NTPs. For this purpose, pure NDP kinase (from baker's yeast) or pure transducin β subunits were incubated with either GDP and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or ADP and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$; the formation (via the Ping-Pong mechanism [15,16]) of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ respectively was monitored. As expected, NDP kinase catalysed the transphosphorylation of NDPs to form NTPs (Figure 13). However, the pure β subunit failed to mediate such a reaction, suggesting that, unlike NDP kinase [15,16], the β subunit was not phosphorylated (as described above) and/or cannot transfer its phosphate to free NDPs.

One possible candidate for the putative kinase might be a CK II-like enzyme, since CK II also has the unusual ability to utilize either GTP or ATP as phosphoryl donor [34,35], analogous to the substrate specificity for β subunit phosphorylation. Immunoblots using rat islet secretory granules indicated the presence of CK II in this fraction (additional results not shown). In order to examine further whether a CK II-like enzyme might mediate the phosphorylation of the β subunit, we examined the effects of spermine and heparin, which are frequently used as a stimulator and inhibitor respectively of CK II [34,35]. Spermine (3 mM) markedly stimulated the phosphorylation of the β subunit (2.7–3.0-fold; Figure 14); heparin (both low- and high-molecular mass forms; 5 μ g/ml) completely abolished β subunit phosphorylation in rat islet secretory granules (Figure 14). Similar degrees of stimulation and inhibition of β subunit phosphorylation by spermine and heparin respectively were seen in the rat islet total membrane and insulinoma secretory granule fractions (additional results not shown).

Figure 14 Preliminary characterization of the putative kinase mediating phosphorylation of the β subunit: sensitivity to heparin and spermine

Rat islet secretory granule proteins were phosphorylated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (+ ATP[S] and App[NH]p; lane A). Spermine (3 mM; lane B), or low- (lane C) or high- (lane D) molecular-mass heparin (5 μ g/ml) were present as indicated. Labelled proteins were identified by autoradiography following SDS/PAGE. Data are representative of five experiments using spermine and two experiments each employing both types of heparin.

Figure 15 (a) Identification of α subunits of pertussis toxin-sensitive and -insensitive G-proteins, and (b) immunological detection of the β subunit in the isolated rat insulinoma secretory granule fraction

(a) Isolated rat insulinoma secretory granule proteins were separated by SDS/PAGE and transferred on to a nitrocellulose membrane. Following the transfer, membranes were incubated with antisera raised against individual α subunits (see Table 1 for specificity details) and protein bands were identified as described in the text. (b) Following transfer of rat insulinoma secretory granule proteins on to a nitrocellulose membrane (as described above), the membrane was incubated with an antiserum reacting with all trimeric G-protein β subunits. Protein bands in the immune complex were identified colorimetrically as described above.

Identification of heterotrimeric subunits of G-proteins on secretory granules

The data described above using GTP[S] and fluoroaluminate (Figure 9) suggested that intact G-protein trimers (i.e. α , β and γ subunit complexes) are present in the secretory granule fraction, and that an intact trimeric conformation is required for β subunit phosphorylation. To examine the localization of various G-protein subunits in the secretory granules of normal rat islets and insulinoma cells, antisera directed against the α subunits of three pertussis toxin-sensitive (G_{11} , G_{13} and G_o) and one pertussis toxin-insensitive (G_q) heterotrimeric G-proteins were used. Details of the specificity of these antisera are given in Table 1. At least four types of α subunit are present in the insulinoma secretory granule fraction (Figure 15a), albeit apparently in varying degrees of abundance. We confirmed the presence of α subunits of the most abundant (G_q and G_{13}) proteins in the secretory granule fraction of normal rat islets as well (additional results not shown). β subunit(s) were also present in the secretory granule fraction from insulinoma tissue (Figure 15b). We observed one major band (37 kDa) and an additional minor band (apparent molecular mass 36 kDa), which may indicate that β_1 and β_2 isoforms [36] are both present in the secretory granules from normal rat islets and insulinoma tissue.

DISCUSSION

It was originally thought that heterotrimeric G-proteins are associated exclusively with plasma membranes. The present findings add to a growing list of recent observations localizing such proteins in various intracellular organelles, including nuclei, Golgi, secretory granules, mitochondria, and even the cytosolic fraction [37–41]. To the best of our knowledge, however, the current studies demonstrate for the first time the presence and identity of apparently intact and functional heterotrimeric G-proteins on the secretory granules of peptide-hormone-secreting cells. Using antisera directed against α subunits of various pertussis toxin-sensitive and -insensitive heterotrimeric G-proteins, the α subunits of at least four heterotrimeric G-proteins were observed on the secretory granules of rat islets and insulinoma cells. These data, along with the immunological identification of β subunits in the cognate fractions, suggest that intact heterotrimeric G-proteins might be present in these fractions (Figure 15). These data extend our recent observations suggesting that a pertussis toxin-sensitive, high-affinity GTPase (presumably intrinsic to a heterotrimeric G-protein) is present on the secretory granules of normal rodent and human β cells and is regulated by positive (e.g. mastoparan or arachidonic acid) or negative (e.g. prostaglandin E_2 , clonidine or adrenaline) modulators of insulin secretion [6,9]. Furthermore, in the current studies, pretreatment with GTP[S] or fluoroaluminate inhibited phosphorylation of the β subunit, also indicating that the intact $\alpha\beta\gamma$ trimer is present in the secretory granule fraction.

Our studies describe a possible novel mechanism for the activation of G-proteins, as defined by the formation of their GTP-bound configuration, in the membrane and secretory granule fractions. The data suggest that the β subunit is phosphorylated transiently, probably at a histidine residue, and that this high-energy phosphate is then transferred via a Ping-Pong mechanism to the GDP bound to G-proteins, thereby enabling the protein to assume the GTP-bound (active) configuration. Furthermore, these data clearly indicate that the β subunit is phosphorylated in GTP-specific manner. The ATP (or ATP[S])-dependent phosphorylation (or dephosphorylation) may be mediated via conversion of these nucleotides into GTP (or GTP[S]), which is catalysed by endogenous islet NDP kinase activity [16]. This formulation was further supported by the fact that ATP (or GTP)-dependent phosphorylation of the β subunit (see the Results section) was inhibited by UDP, an inhibitor of NDP kinase [16]. While there have been several studies depicting the GTP-specific phosphorylation of unidentified proteins in the molecular mass region 30–40 kDa [42–44], to our knowledge there is only one report [18] describing specifically the phosphorylation of a G-protein β subunit in mammalian cells. Wieland et al. [18] have reported the GTP-specific phosphorylation of the β subunit on a histidine residue using HL 60 cell membranes, in close agreement with our observations using β cell subcellular fractions.

The β subunit could not be autophosphorylated (even in the presence of the α subunit) but required the addition of membranes, suggesting that a membrane-associated (histidine) kinase might be involved. Based upon its use of GTP as well as ATP as a phosphoryl donor, its stimulation by spermine and its inhibition by heparin, this enzyme may have functional similarities to CK II. This formulation is further supported by the immunological (present study) and functional [45] characterization of CK II activity in islets, including the secretory granule fractions (A. Kowluru and S. A. Metz, unpublished work). However, this conclusion would not be in accordance with classical CK II-mediated phosphorylation reactions, since

CK II has been reported to phosphorylate only serine and threonine residues [31,32]. In the present study, we tentatively identified the phosphoamino acid on the β subunit as histidine, based on the its sensitivities to acid pH, heat, DEPC and hydroxylamine. Interestingly, however, our studies (Figure 6) demonstrated a modest loss of labelling from the phosphorylated β subunit under alkaline pH conditions. This finding argues in favour of an additional phosphorylation site(s) on the β subunit. Therefore it is possible that CK II or a similar enzyme phosphorylates the β subunit on a serine residue, which in turn is transferred to histidine via an intramolecular transfer mechanism [46]. Engel et al. [47] have recently reported the phosphorylation by CK II of a serine residue on NDP kinase, leading to modification of the substrate binding and catalytic properties of the enzyme. Thus, alternatively, CK II might phosphorylate NDP kinase (indigenous to β cells [16]) which, in turn, then transfers its phosphate to the β subunit in a kinase cascade. The latter possibility is in accordance with the inhibition of β subunit phosphorylation by UDP, an inhibitor of NDP kinase activity [15,16]. The activity of NDP kinase could also explain the inability of the putative β subunit kinase to use $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as a substrate in the presence of UDP (i.e. ATP was active only by transfer of phosphate to GDP to yield the selective substrate $[\gamma\text{-}^{32}\text{P}]\text{GTP}$). Parenthetically, the stimulation of this putative kinase by spermine may be relevant to the stimulatory effects of the latter on insulin synthesis and secretion in islets [48]. Additional studies will clearly be needed to isolate and characterize the kinase, or cascade of kinases, involved in this reaction.

In summary, these studies identify α subunits of both stimulatory and inhibitory classes of heterotrimeric G-proteins on the secretory granules of normal rat islets as well as pure insulinoma cells. These G-proteins may be indigenous to secretory granules derived from the β cell component of the islet, since rat insulinoma tissue is comprised of > 95 % β cells. These studies also describe a similar subcellular localization for β subunits (and presumably γ subunits, since β subunits only exist in close association with γ subunits [1,2,23]). The β subunit, after its phosphorylation, appears to be able to transfer its high-energy phosphate to $G_{z\text{-GDP}}$ to yield the active configuration. It is tempting to speculate that one (or more) of these proteins may comprise G_E or G_{EI} , the elusive G-proteins postulated to respectively stimulate or inhibit exocytosis [49].

These studies were supported by the Veterans Administration (to A.K. and S.A.M.) and the National Institutes of Health (DK37312 to S.A.M. and DK47919 to C.J.R.). We thank James Stephens for excellent technical help. We thank Dr. P. Robertson and Dr. H.-J. Zhang for HIT cells, Dr. D. Scharp for human islets, Dr. C. Wollheim for INS-1 cells and Dr. A. Yamazaki for transducin α and β/γ subunits and helpful discussions. We thank Dr. Guodong Li for providing assistance in culturing HIT and INS-1 cells.

REFERENCES

- 1 Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M. and Satoh, T. (1991) *Annu. Rev. Biochem.* **60**, 349–400
- 2 Birnbaumer, L., Abramowitz, J. and Brown, A. M. (1990) *Biochim. Biophys. Acta* **1031**, 163–224
- 3 Prentki, M. and Matschinsky, F. M. (1987) *Physiol. Rev.* **67**, 1185–1248
- 4 Metz, S. A. (1991) *Diabetes* **40**, 1565–1573
- 5 Metz, S. A., Rabaglia, M. E., Stock, J. B. and Kowluru, A. (1993) *Biochem. J.* **295**, 31–40
- 6 Kowluru, A. and Metz, S. A. (1994) *Biochem. J.* **297**, 399–406
- 7 Regazzi, R., Kikuchi, A., Takai, Y. and Wollheim, C. B. (1992) *J. Biol. Chem.* **267**, 17512–17519

- 8 Kowluru, A. and Metz, S. A. (1994) in *Molecular Biology of Diabetes* (Draznin, B. and LeRoith, D., eds.), pp. 249–283, Humana Press Inc., Totowa, NJ
- 9 Kowluru, A., Rabaglia, M. E., Muse, K. E. and Metz, S. A. (1994) *Biochim. Biophys. Acta* **1222**, 348–359
- 10 Li, G., Regazzi, R., Roche, E. and Wollheim, C. B. (1993) *Biochem. J.* **289**, 379–385
- 11 Metz, S. A., Rabaglia, M. E. and Pintar, T. J. (1992) *J. Biol. Chem.* **267**, 12517–12527
- 12 Metz, S. A., Meredith, M., Rabaglia, M. E. and Kowluru, A. (1993) *J. Clin. Invest.* **92**, 872–882
- 13 Takai, Y., Kaibuchi, K., Kikuchi, A. and Kawata, M. (1992) *Int. Rev. Cytol.* **133**, 187–235
- 14 Kikkawa, S., Takahashi, K., Takahashi, K.-I., Shimada, N., Ui, M., Kimura, N. and Katada, T. (1990) *J. Biol. Chem.* **265**, 21536–21540
- 15 Kimura, N. (1993) *Handb. Exp. Pharmacol.* **108**, 485–498
- 16 Kowluru, A. and Metz, S. A. (1994) *Biochemistry* **33**, 12495–12503
- 17 Randazzo, P. A., Northup, J. K. and Kahn, R. A. (1992) *J. Biol. Chem.* **267**, 18182–18189
- 18 Wieland, T., Nürnberg, B., Ulibarri, I., Kaldenberg-Stasch, S., Schultz, G. and Jakobs, K. H. (1993) *J. Biol. Chem.* **261**, 18111–18118
- 19 Stock, J. B., Ninfa, A. J. and Stock, A. M. (1989) *Microbiol. Rev.* **53**, 450–490
- 20 Hess, J. F., Oosawa, K., Kaplan, N. and Simon, M. I. (1988) *Cell* **53**, 79–87
- 20a Kowluru, A. and Metz, S. A. (1994) *Diabetes* **43** (Suppl. 1), 91A
- 20b Kowluru, A. and Metz, S. A. (1994) *Clin. Res.* **42**, 333A
- 21 Chick, W. L., Warren, S., Chute, R. N., Like, A. A., Lauris, V. and Kitchen, K. C. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 628–632
- 22 Rhodes, C. J., Brennan, S. O. and Hutton, J. C. (1989) *J. Biol. Chem.* **264**, 14240–14245
- 23 Miles, E. W. (1977) *Methods Enzymol.* **47**, 431–442
- 24 Van Etten, R. L., Waymack, P. P. and Rehkop, D. M. (1974) *J. Am. Chem. Soc.* **96**, 6782–6785
- 25 Ovaldi, J., Libor, S. and Elodi, P. (1969) *Biochim. Biophys. Acta* **4**, 365–374
- 26 Hokin, L. S., Sastry, P. S., Galsworthy, P. R. and Yoda, A. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **54**, 177–184
- 27 Yamazaki, A., Tatsumi, M., Torney, D. C. and Bitensky, M. W. (1987) *J. Biol. Chem.* **262**, 9316–9323
- 28 Draznin, B., Chang, L., Wayne Leitner, J., Takata, Y. and Olefsky, J. M. (1993) *J. Biol. Chem.* **268**, 19998–20001
- 29 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 30 Kowluru, A. and Metz, S. A. (1994) *Clin. Res.* **42**, 333A
- 31 Bominaar, A. A., Molijn, A. C., Pestel, M., Veron, M. and Van Haastert, P. J. M. (1993) *EMBO J.* **12**, 2275–2279
- 32 Wieland, T., Ulibarri, I., Gierschik, P. and Jakobs, K. H. (1991) *Eur. J. Biochem.* **196**, 707–716
- 33 Wieland, T., Ronzani, M. and Jakobs, K. H. (1992) *J. Biol. Chem.* **267**, 20791–20797
- 34 Hathaway, G. M. and Traugh, J. A. (1982) *Curr. Top. Cell. Regul.* **21**, 101–127
- 35 Tuazon, P. T. and Traugh, J. A. (1991) *Adv. Second Messenger Phosphoprotein Res.* **23**, 143–164
- 36 Hirsch, K.-D., Tychowiecka, I., Gausepohl, H., Frank, R., Rosenthal, W. and Schultz, G. (1989) *Biochim. Biophys. Acta* **1013**, 60–67
- 37 Wilson, B. S., Komuro, M. and Farquhar, M. G. (1994) *Endocrinology* (Baltimore) **134**, 233–244
- 38 Lewis, J. M., Woolkalis, M. J., Gerton, G. L., Smith, R. M., Jarett, L. and Manning, D. R. (1991) *Cell Regul.* **2**, 1097–1113
- 39 Bokoch, G. M., Bickford, K. and Bohl, B. P. (1988) *J. Cell Biol.* **106**, 1927–1936
- 40 Watson, E. L., DiJulio, D., Kauffman, D., Iversen, J., Robinovitch, M. R. and Izutsu, K. T. (1992) *Biochem. J.* **285**, 441–449
- 41 Phillips, M. R., Staud, R., Pillinger, M., Feoktistov, A., Volker, C., Stock, J. B. and Weissman, G. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 2283–2287
- 42 Amir-Zaltsman, Y., Extra, E., Walker, N., Lindner, H. R. and Salomon, Y. (1980) *FEBS Lett.* **122**, 166–170
- 43 Dawson, A. P., Comerford, J. G. and Fulton, D. V. (1986) *Biochem. J.* **234**, 311–315
- 44 Hedge, A. N. and Das, M. R. (1990) *Mol. Cell. Biol.* **10**, 2468–2474
- 45 Thams, P., Capito, K. and Hedekov, C. J. (1986) *Diabetologia* **29**, 888–892
- 46 Wei, Y.-F. and Mathews, H. R. (1991) *Methods Enzymol.* **200**, 388–395
- 47 Engel, M., Issinger, O.-G., Lascu, I., Seib, T., Dooley, S., Zang, K. D. and Welter, C. (1994) *Biochem. Biophys. Res. Commun.* **199**, 1041–1048
- 48 Sjöholm, Å., Arkhammar, P., Welsh, N., et al. (1993) *J. Clin. Invest.* **92**, 1910–1917
- 49 Gomperts, B. D., Churcher, Y., Koffer, A., Kramer, I. M., Lillie, T. and Tatham, P. E. R. (1990) *Biochem. Soc. Symp.* **56**, 85–101