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Guanosine 5′-(γ-thio) triphosphate (GTPγS) inhibits phosphorylation of insulin receptor and a novel GTP-binding protein, G_{ir}, from human placenta

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Abstract

A novel 66 kDa GTP-binding protein, designated G_{ir}, has been partially purified along with insulin receptor (IR) from human placenta. This protein binds 8-azido-GTP, is ADP-ribosylated by pertussis toxin, phosphorylated by IR tyrosine kinase and cross-reacts with antibodies against synthetic peptides from the GTP-binding domain of $G_{z\alpha}$ (P960). Phosphorylation of IR-β subunit and G_{ir} by IR tyrosine kinase was almost completely inhibited by 100 μM GTPγS, >75% by 50 μM and 20–30% by 1 μM, while GDP at these concentrations had no significant effect on the phosphorylation. IR tyrosine kinase phosphorylated G_{ir} at the tyrosine residues. These studies indicate regulation of IR tyrosine kinase activity by guanosine phosphates and involvement of G_{ir} in insulin action.

Key words: Insulin receptor; G-Protein; Signal transduction; Phosphorylation

1. Introduction

Cellular events of insulin action begin with binding of insulin to the α-subunits of insulin receptor (IR). Such binding produces conformational changes in the transmembranous IR-β subunits, activating their tyrosine kinase activity, which phosphorylates both IR-β subunits and other proteins as well [1,2]. This sequence of events is widely believed to represent one mechanism by which insulin's signals are transduced. Furthermore, it has been proposed that insulin-induced aggregates of IR have severalfold higher tyrosine kinase activity as compared to native (tetrameric) IR [3]. Although binding of insulin to IR and activation of its tyrosine kinase are well understood, subsequent steps in insulin signal transduction from IR to its effector(s) are not known. However, there is indirect evidence to support the involvement of GTP-binding protein(s) which could mediate or relay insulin's signal across the cell membrane.

In recent years many approaches have been made to identify G-protein(s) involved in insulin's action. A number of investigators have used bacterial toxins (cholera and pertussis toxins) which are known to ADP-ribosylate some of the G-proteins and alter their interactions with the receptors. Cholera toxin, which ADP-ribosylates primarily the stimulatory G-proteins (G_s), has been shown to increase insulin-stimulated protein synthesis [4], and insulin inhibits stimulation of adenylate cyclase by this toxin [5]. Pertussis toxin, which ADP-ribosylates the inhibitory type of G-proteins (G_i) and G_o, inhibits a number of insulin-stimulated cellular events such as glucose transport [6] and its metabolism [7], protein synthesis [4], and activation of cyclic AMP phosphodiesterase [8]. Interestingly, insulin inhibits pertussis toxin catalyzed ADP-ribosylation of G_i [9] and this toxin also attenuates insulin-induced inhibition of adenyl cyclase [8]. We have recently isolated a novel 66 kDa GTP-binding protein, G_{ir}, from human placenta and have shown that G_{ir} co-purifies with IR through DEAE-Sephacel and WGA-Agarose chromatography steps [11–13]. The G_{ir} binds GTPγS and 8-azido-GTP in the presence of Mg^{2+}, has GTPase activity, is ADP-ribosylated by pertussis toxin, co-purifies with IR, and is phosphorylated by the insulin-stimulated IR tyrosine kinase. Furthermore, we have demonstrated that addition of GTPγS to IR-G_{ir}-fraction reduces insulin-binding to IR, and that addition of insulin increases the binding of GTPγS to G_{ir} by 3- to 5-fold [12]. Other evidence implicating G-proteins in insulin's action is the recent demonstration that a non-hydrolyzable GTP analog (GTPγS) slightly inhibits (22%) the insulin-induced phosphorylation of IR-β subunit and significantly (65%) the phosphorylation of other substrates using purified membranes [10]. Decreased autophosphorylation of the IR-β subunit has been attri-

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buted to decreased insulin-binding to IR in the presence of GTPγS. Jo et al. [14] have further demonstrated that an insulin receptor peptide stimulates GTPγS binding to a 67 kDa GTP-binding protein from human placenta. We now report almost complete inhibition of phosphorylation of IR-β subunit and G_{ir} by GTPγS, and insulin-induced phosphorylation of G_{ir} at tyrosine residues(s) by IR.

2. Materials and methods

2.1. Chemicals

Antibodies against a synthetic peptide from the GTP-binding region of G_{zz} (P960, sequence GTSNSGKSTIVQMK) which cross-react with a number of other G_α-subunits, $\alpha_o > \alpha_i > \alpha_t > \alpha_s > \alpha_z$ [15], were the kind gift from Dr. A.G. Gilman of The University of Texas Health Science Center at Dallas. Monoclonal antibodies against phosphotyrosine-linked to Agarose were purchased from Sigma Chemical Co. [γ-^{35}S]GTPγS, [γ-^{32}P]ATP and [α-^{32}P]NAD were purchased from New England Nuclear Division of DuPont. [γ-^{32}P]8-azido-GTP was purchased from ICN, Canada Ltd. Pertussis toxin was purchased from List Biochemicals, California. DEAE-Sepharose, Wheat Germ Agglutinin (WGA)-Sepharose, CHAPS, guanine nucleotides (GDP, GTP and GTPγS), trichloro acetic acid (TCA), trifluoro acetic acid (TFA), Ponsceau-S, TPCK-treated trypsin, polyvinyl pyrrolidone-40 (PVP-40), phosphoserine, phosphothreonine, and phosphotyrosine were obtained from Sigma Chemical Co., St. Louis, MO. Acetonitrile and isopropanol were purchased from Aldrich, Milwaukee, WI. Polyvinylidene difluoride (PVDF) membrane and Pre-coated thin layer chromatography (TLC) plates were obtained from Bio-Rad, Richmond, CA. and Sybron/Brinkman Instruments, New York, NY, respectively. [^{125}I]Insulin was a gift from Dr. Charles C. Stuart of The University of Texas Medical Branch, Galveston, Texas.

2.2. Isolation of G_{ir} and IR

Human placentae were obtained within 4 h of delivery and parenchymal tissue was dissected free of chorion, amnion, umbilical cord and large blood vessels. Unless otherwise specified, all the procedures were carried out at 4°C and all the solutions used in the purification contained 100 μM phenylmethylsulfonylfluoride (PMSF), 0.25 M sucrose and 0.15 M NaCl. The parenchymal tissue was homogenized in 3 volumes of 25 mM Tris-HCl, pH 7.4 containing 1 mM $CaCl_2$ using a Polytron (Kinamatica-GmbH, setting no. 4) for 2 min. The homogenate was centrifuged at 10,000 × g for 30 min using a Sorvall RC5-B high speed centrifuge. The supernatant was fortified with $MgSO_4$ (0.2 mM) and centrifuged at 30,000 × g for 60 min. The membrane pellet was homogenized using a Potter-Elvehjem glass homogenizer in 25 mM Tris-HCl, pH 7.4 containing 1 mM $CaCl_2$ and centrifuged at 30,000 × g for 60 min. The pellet was washed once more and stored at −70°C. The membrane preparation was thawed and suspended in 10 volumes of 25 mM Tris-HCl, pH 7.4 containing a final concentration of 5 mM EGTA, resuspended in a Polytron for 30 s and centrifuged at 30,000×g for 60 min.

For solubilization of membrane protein, the pellet was suspended in 10 volumes of buffer A (50 mM HEPES-NaOH, pH 7.4, 0.25 M sucrose, 0.15 M NaCl, 100 μM PMSF) containing 1% CHAPS. After 45 min of vigorous stirring at 4°C, the sample was centrifuged at 30,000 × g for 60 min and the supernatant was diluted 10-fold with buffer A. The pellet was extracted once more following the same procedure. The extracts were pooled and immediately applied to a DEAE-Sephacel column (2.5 × 20 cm), equilibrated with buffer A containing 0.1% CHAPS at a flow rate of 12 ml/h. The column was washed with 200 ml of equilibrating buffer containing 500 mM NaCl. This resulted in the elution of a significant amount of GTP-binding proteins. As observed by previous investigators [16], this may represent several well characterized G-proteins (G_s, G_i and G_p). After washing the column thoroughly with 200 ml of buffer A containing 1 M NaCl, elution of IR and G_{ir} was achieved by buffer A containing 0.1% Triton X-100 and 1 M NaCl. Fractions that contained both GTPγS-binding and insulin-binding activity were pooled, dialyzed against buffer A, and applied to a 10 ml WGA-Sepharose affinity column, equilibrated with buffer A containing 0.1% Triton X-100. Insulin receptor and G_{ir} co-eluted with the equilibrating buffer containing 0.3 M *N*-acetylglucosamine. Each fraction (5 ml) was analyzed for GTPγS-binding and insulin-binding activity. The fractions that had GTPγS-binding activity also had insulin-binding activity. These fractions were pooled and dialyzed extensively against equilibrating buffer. Unless otherwise specified, this fraction (IR-G_{ir}- fraction) was used for all the studies described here.

2.3. [^{35}S]GTPγS and [^{125}I]insulin-binding assays

[^{35}S]GTPγS-binding to G-Proteins was determined as described by Evans et al. [16] and [^{125}I]insulin-binding to IR was determined according to the method described by Fugita-Yamaguchi et al. [17].

2.4. Phosphorylation of the IR-G_{ir}-fraction and immunoprecipitation

Phosphorylation of the IR-G_{ir}-fraction was performed in the absence and presence of insulin (100 nM) in a reaction mixture containing 5 mM $MnCl_2$, 16 μM [γ-^{32}P]ATP, 60,000 cpm/pmol as described by Krupinski et al. [18]. The reaction was stopped by the addition of 5 × stop-buffer (50 mM HEPES-NaOH, pH 8.0, 2 mM EDTA, 50 mM sodium pyrophosphate, 10 mM sodium fluoride and 10 mM ATP). Subsequently, antiphosphotyrosine-Agarose was added (1:1) and incubated overnight. Unbound proteins were removed by washing with phosphate-buffered saline (20 mM phosphate, pH 7.4 and 0.15 M NaCl) containing 0.1% NP-40. Phosphorylated and Agarose-bound proteins were solubilized in 5 × Laemmli sample buffer [19] and separated by 7.5% SDS-PAGE under reducing conditions (5 mM β-mercaptoethanol). 8-Azido-GTP-binding [12], and ADP-ribosylation of G-proteins by Pertussis toxin [20], slab gel electrophoresis [19], and autoradiography were performed as described previously [12,16,18]. Western blot analysis using antibodies P960 was performed following the methods of Casey et al. [15].

2.5. Determination of phosphorylated amino acid residue(s)

Placental membrane and detergent-solubilized membrane extract (1 ml) were activated by pre-incubation with 100 nM insulin [17]. Phosphorylation was performed by incubating the insulin-activated sample with [^{32}P]ATP for 30 min. at room temperature and precipitated with TCA (10% final, v/v) overnight at 4°C. The precipitate was centrifuged at 14,000×g for 15 min at 4°C, washed twice with 2 volumes of ethyl ether, resuspended in Laemmli's buffer, resolved on a 7.5% reducing SDS-PAGE, and electroblotted onto a PVDF membrane. The membrane was stained for 1–2 min with 0.1% Ponceau-S (in 1% acetic acid), washed quickly with water twice, neutralized immediately by washing with 200 μM NaOH for 1–2 min, dried and wrapped in saran wrap, and kept at −70°C for autoradiography.

Five to six phosphorylated 66 kDa bands were carefully cut out, pooled in a microfuge tube, and blocked in 500 μl of 0.5% PVP-40 dissolved in 100 mM acetic acid (to prevent adsorption of protease to the membrane during digestion) for 30 min at 37°C. Excess PVP-40 was removed by extensive washing with water (5–6 changes). The 66 kDa protein bands were cut into smaller pieces of approximately 1 mm × 1 mm, put back into the same tube and were subjected to trypsin digestion (enzyme to substrate ratio, 1 : 20) in 200 μl of 100 mM $NaHCO_3$, pH 8.2/acetonitrile, 95:5 (v/v), at 37°C overnight. After the digestion the reaction mixture was lyophilized, resuspended in 500 μl of 6 N HCl and hydrolyzed under vacuum for 3 h at 110°C. The acid-hydrolyzed digest was diluted 4-fold with distilled water, lyophilized, resuspended in 50 μl of trifluroacetic acid (TFA)/H_2O [10:90 (v/v)], and immediately applied to a narrow bore (2.1 mm i.d.) reverse-phase HPLC column (ODS/C_{18}). The phosphorylated amino acid residues were eluted with a 0–60% linear gradient of acetonitrile containing 0.1% TFA. Elution profiles of 20 μg each of phosphoserine (p-ser), phosphothreonine (p-thr) and phosphotyrosine (p-tyr) were obtained under the similar elution conditions.

The eluted fractions containing radioactivity and corresponding to phosphotyrosine (p-tyr) peak were collected, lyophilized, resuspended in 50 μl of HPLC grade water, and applied to a pre-irrigated thin layer chromatography plate along with standards containing phosphoamino acids; p-ser(S), p-thr(T), and p-tyr(Y). The phosphorylated amino acids were resolved by three irrigation cycles with resolving solvent (propionic acid/2-isopropanol/water (20:70:10)) for 8 h each, followed by air drying. The TLC plates were developed by ninhydrin spray (0.33%

ninhydrin in ethanol), followed by heating at 60°C for 1 h. Finally, the plate was wrapped with saran wrap and stored overnight at −70°C for autoradiography.

2.6. Effect of GTPγS on the phosphorylation of IR-β subunit and G_{ir}

The IR-G_{ir}-fractions were first incubated with various concentrations of GDP and GTPγS in Tris buffer (50 mM Tris-HCl, pH 7.4 containing 0.1% Triton X-100 and 100 μM PMSF). Subsequently, insulin (100 nM) was added to one set and to the other set equivalent amount of Tris-buffer was added. The samples were further incubated for 1 h at 25°C, phosphorylated as described above, and subjected to 7.5% SDS-PAGE under reducing conditions. The gels were dried and subjected to autoradiography [11].

3. Results

Approximately 70% of total G-Proteins were eluted from the DEAE-Sephacel column with buffer A containing 0.1% CHAPS and 0.5 M NaCl and the remaining 30% eluted along with IR (as determined by [^{35}S]GTPγS and [^{125}I]insulin-binding) with 1 M NaCl in buffer A containing 0.1% Triton X-100. Almost all the IR and GTPγS-binding activity, eluted from DEAE-Sephacel column with 1 M NaCl, adsorbed on WGA-Agarose and co-eluted with 0.3 M *N*-acetylglucosamine [12]. This observation indicated that either G_{ir} and some other GTP-binding proteins are complexed with receptors which are glycosylated, for example IR, or that some of the GTP-binding proteins may contain carbohydrate moieties. In the WGA-fraction, 8-azido-GTP bound to 66 kDa, 45 kDa, and 41 kDa proteins (Fig. 1, lanes 1,2). However, ADP-ribosylation in the presence of pertussis toxin indicated two major substrates (Fig. 1, lanes 3,4), one of 41–43 kDa and the other 66 kDa (G_{ir}). Similarly, antibodies against GTP-binding domain (P960) demonstrated two cross-reacting protein bands of 66 kDa and approximately 41 kDa (Fig. 1, lane 5). Antibodies P960 absorbed by synthetic peptide P960 (corresponding to the GTP-binding domain of $G_{z\alpha}$), did not cross-react with any protein on western blot (data not shown).

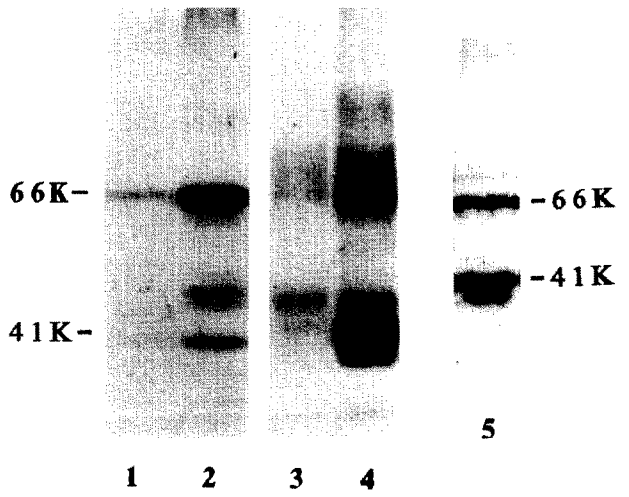

Fig. 1. Characterization of placental G_{ir}. The WGA-Sepharose fraction containing IR-G_{ir} was used for 8-azido-GTP-binding in the presence of 200 μM ATP without (lane 1) and with (lane 2) exposure to UV light; for ADP-ribosylation in the absence (lane 3) and presence (lane 4) of pertussis toxin; and for Western blot analysis (lane 5) using antiserum P960. A 10% gel was used for SDS-PAGE.

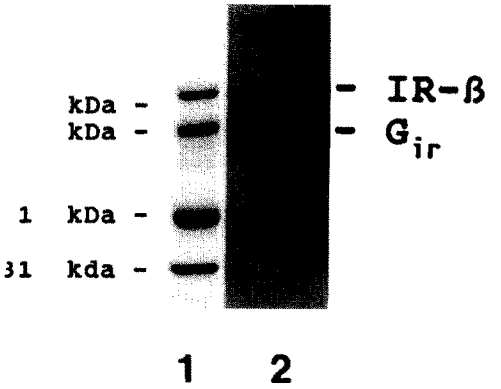

Fig. 2. Immunoprecipitation of phosphorylated IR-β and G_{ir}. Lane 1 shows standard molecular weight markers. Lane 2 shows IR-β and G_{ir} immunoprecipitated with monoclonal antiphosphotyrosine antibodies (autoradiograph).

Insulin-activated IR tyrosine kinase phosphorylated IR-β subunit as well as 66 kDa protein. The phosphorylation of G_{ir} occurred at the tyrosine moiety as shown by the precipitation of the phosphorylated G_{ir} by monoclonal antibodies against phosphotyrosine (Fig. 2). Furthermore, acid hydrolysis of ^{32}P-phosphorylated G_{ir}, transferred on PVDF after SDS-PAGE, followed by the separation of phosphorylated amino acids by reverse-phase high-performance liquid chromatography and analysis by TLC indicated that the phosphorylation occurred at the tyrosine moiety (Fig. 3).

Phosphorylation of both IR-β subunit and G_{ir} was inhibited >75% by 50 μM and almost completely by 100 μM GTPγS both in the absence or presence of 200 nM insulin (Fig. 4, top and bottom panels). Even 1 μM GTPγS inhibited insulin-induced phosphorylation by 20–30%. On the other hand, 100 μM GDP had no significant effect on the phosphorylation of IR and G_{ir} (data not shown).

4. Discussion

Thus far two distinct families of hormone signal transducing G-Proteins have been characterized, 'low molecular weight G-proteins' comprising only a single 20–28 kDa polypeptide, and 'classical heterotrimeric G-proteins' consisting of α, β, and γ subunits [21–23]. The G_{α}-subunit binds guanine nucleotides (GTP and GDP), has GTPase activity, some types can be ADP-ribosylated by pertussis or cholera toxin, and can be phosphorylated by IR tyrosine kinase in the presence of biological membranes or liposomes [18]. There is a distinct diversity in the size of α-subunits of the G-proteins, while β- and γ-subunits show a lesser degree of size variability [21].

G_α-subunits have a molecular weight of 39,000 to 54,000, however, a 74 kDa GTP-binding protein (G_h) associated with α_1-adrenergic receptors has also been isolated recently [24]. Our simultaneous demonstration of a 66 kDa protein (G_{ir}) in human placenta which exhibits all the properties of a G_α-subunit, except size, further shows the presence of higher molecular weight GTP-binding proteins.

Since G_{ir} has GTPase activity (11), it was not possible to study the effect of GTP on the phosphorylation of IR and G_{ir}. Instead, GTPγS which is not hydrolysed by GTPase was used. Differential inhibition of insulin receptor phosphorylation by GTPγS and GDP may indicate a regulatory role for guanine nucleotide(s) assuming that the effect of GTP and GTPγS is similar. The GTPγS concentration that almost completely inhibits IR and G_{ir} phosphorylation in vitro is within the physiological range of GTP [25]. We have shown earlier that 100 μM GTPγS significantly inhibits insulin-binding to IR-G_{ir}-fraction [12], whereas Davis and McDonald [10] have demonstrated that GDP increases insulin-binding to IR. Furthermore, prior binding of insulin to IR-G_{ir}-fraction increases high affinity binding of GTPγS by 3- to 5-fold [12]. Therefore, it is reasonable to speculate that in vivo, insulin binding to IR-α subunits would stimulate GTP-binding to G-proteins and activate IR-β tyrosine kinase which in turn could phosphorylate G_{ir} and other G-proteins. GTP would decrease insulin binding to IR and $G_{ir.GTP}$ could be hydrolysed to the GDP-bound form ($G_{ir.GDP}$) which would increase insulin binding and would not affect phosphorylation of the IR tyrosine kinase.

Jo et al. [26] have also identified and partially purified two distinct GTP-binding proteins (67 and 41 kDa) that are associated with IR. The dissociation of these GTP-binding proteins from the IR results in loss of insulin-stimulated IR kinase activity which could be restored upon the addition of the former. The 67 kDa protein, which appears to be similar to our 66 kDa GTP-binding protein [11–13], could further be purified over the GDP-agarose column, binds 8-azido-GTP and cross-reacts with antibodies [15] specific to G_α subunit. The 41 kDa protein has characteristics of G_i-type protein and can be ADP-ribosylated in the presence of pertussis toxin. Recently, it has been shown that IR-β subunit also possesses two distinct binding/activating domains for GTP-binding proteins [14] similar to that of IGF-II/mannose G-phosphate receptor [27]. It has been reported that a synthetic peptide specific for one of these two domains, $GPBP_2$, 1135–1156 (G-proteins binding peptide, 1135–1156) increases GTPγS binding to the IR associated G-proteins by severalfold [14]. Cocozza et al. have suggested that a natural mutation of Arg → Gln at codon 1152 (represented in $GPBP_2$, 1135–1156) in the exon 20 of IR results into impaired IR kinase activity and could contribute to the insulin-resistant status in NIDDM patients [28]. In purified IR preparations, ATP binds directly to IR [29] and brings about conformational change in IR-β [30] leading to its autophosphorylation. Guanine nucleotides (GTP/GMP-PNP) do not interfere in or

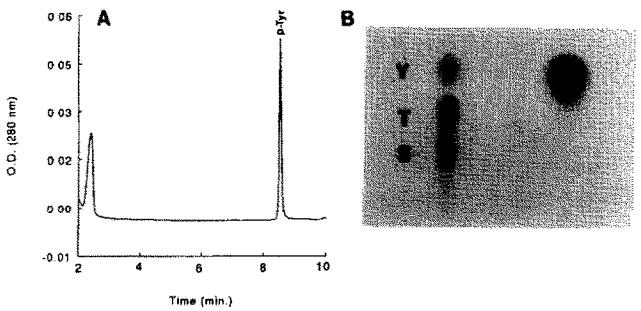

Fig. 3. Identification of phosphorylated tyrosine residues of G_{ir}. (A) High-performance liquid chromatography profile of phosphotyrosine standard (20 ng), (B) thin layer chromatography profile of phsorphorylated G_{ir}. The left lane shows the separation of a mixture of standards [containing P-Ser(S), P-Thr(T) and P-Tyr(Y)] stained with ninhydrin. The right lane shows the radiolabeled [^{32}P]phosphotyrosine residues in acid-hydrolysed, HPLC-separated phosphorylated G_{ir}.

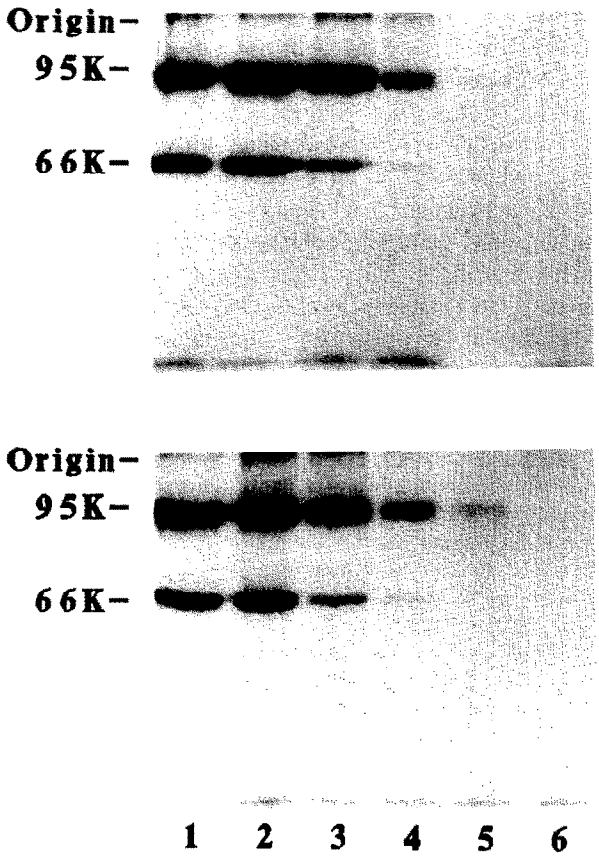

Fig. 4. Effect of GTPγS on the phosphorylation of insulin receptor β-subunit and G_{ir}. DEAE-Sephacel fraction containing IR-G_{ir} was used for phosphorylation without (upper panel) and with (lower panel) 200 nM insulin pre-incubation with at 25°C [16]. Samples after incubation with or without insulin were further incubated with GTPγS: none, (1); 1 μM (2); 10 μM (3); 50 μM (4); 100 μM (5); and 500 μM (6). Subsequently, phosphorylation mixture was added as described in the text. Afterwards proteins were separated on SDS-PAGE, stained with Coomassie blue and subjected to autoradiography.

compete for ATP-binding to IR [29]. It is therefore likely that GTPγS binds to the G-proteins and not to IR. If IR and G-protein(s) are complexed, binding of GTPγS to G_{ir} would in turn affect properties of IR including insulin binding and IR tyrosine kinase activity.

In conclusion, G_{ir} could play a role in at least some of insulin's actions because: the 66 kDa GTP-binding protein, G_{ir}, may be complexed with IR; insulin activates GTPγS-binding to G_{ir}; IR tyrosine kinase phosphorylates G_{ir} and insulin stimulates it, GTPγS inhibits insulin-binding to IR while GDP stimulates it, and GTPγS (equivalent to physiological concentrations of GTP) almost completely blocks phosphorylation of both IR-β subunit and G_{ir}. Further studies are in progress to elucidate the role of G_{ir} in insulin signal transduction.

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