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Ligand-dependent autophosphorylation of the insulin receptor is positively regulated by G_i-proteins

J. KREUZER*, B. NÜRNBERG† and H. I. KRIEGER-BRAUER*[1]

*Innere Medizin III, Universität Heidelberg, Bergheimer Str. 58, D-69115, Heidelberg, Germany, and †Institut für Biochemie und Molekularbiologie II, Heinrich-Heine-Universität, Düsseldorf, Germany

Previously, we have shown that the human insulin receptor (IR) interacts with G_i2, independent of tyrosine kinase activity and stimulates NADPH oxidase via the Gα subunit of G_i2. We have now investigated the regulatory role of G_i2-proteins in IR function. For the experiments, isolated IRs from plasma membranes of human fat cells were used. The activation of IR autophosphorylation by insulin was blocked by G-protein inactivation through GDPβS (guanosine 5′-[β-thio]disphosphate). Consistently, activation of G-proteins by micromolar concentrations of GTPγS (guanosine 5′-[γ-thio]triphosphate) induced receptor autophosphorylation 5-fold over baseline and increased insulin-induced autophosphorylation by 3-fold. In the presence of 10 μM GTPγS, insulin was active at picomolar concentrations, indicating that insulin acted via its cognate receptor. Pretreatment of the plasma membranes with pertussis toxin prevented insulin- and GTPγS-induced autophosphorylation, but did not disrupt the IR–G_i2 complex. The functional nature of the IR–G_i2 complex was made evident by insulin's ability to increase association of G_i2 with the IR. This leads to an augmentation of maximal receptor autophosphorylation induced by insulin and GTPγS. The specificity of this mechanism was further demonstrated by the use of isolated preactivated G-proteins. Addition of $G_i2\alpha$ and G$\beta\gamma$ mimicked maximal response of insulin, whereas Gαs or Gαo had no stimulatory effect. These results define a novel mechanism by which insulin signalling mediates tyrosine kinase activity and autophosphorylation of the IR through recruitment of G_i-proteins.

Key words: autophosphorylation, G-protein, insulin receptor, manganese (II).

INTRODUCTION

The IR (insulin receptor) is an $\alpha2\beta2$ heterotetrameric glycoprotein possessing intrinsic protein-tyrosine kinase activity [1]. On insulin binding to the α-subunit, the IR undergoes a poorly characterized conformational change that results in autophosphorylation of specific tyrosine residues within the cytoplasmic portion of the β-subunits. Three regions in the β-subunit are sites of autophosphorylation: (i) the juxtamembrane region, (ii) the activation loop within the tyrosine kinase domain and (iii) the C-terminal tail. Autophosphorylation of specific tyrosine residues stimulates the receptor's catalytic activity and creates recruitment sites for downstream signalling molecules such as the IR substrate proteins and Shc.

Activation of tyrosine kinase activity is one of the earliest steps in insulin action and may be essential for many of insulin's biological effects [2]. Recently, it was demonstrated by us and others that heterotrimeric G-proteins, typical transducers of seven-helix receptors, are also associated with the IR and involved in insulin signalling [3–6]. Even though several studies have suggested that this association participates in the regulation of IR autophosphorylation, the supporting results so far are not conclusive. There are controversial reports concerning the effect of the G-protein activator, GTPγS (guanosine 5′-[γ-thio]triphosphate), on IR autophosphorylation. For instance, it was shown that GTPγS increases the autophosphorylation of partially purified cardiac IRs [7]. In contrast, an inhibition of IR kinase activity by GTPγS has been observed in IR preparations from rat adipocytes [8,9]. The underlying reason for this discrepancy could be the conditions used for the experiments.

Investigations of the IR tyrosine kinase in cell-free preparations are generally performed in the presence of supraphysiological concentrations of $MnCl_2$, which are supposed to be essential for ligand-induced activation of the receptor [10]. This, however, may be very problematic, as earlier studies have shown that bivalent cations such as $MnCl_2$ obscure an involvement of G-proteins in receptor signalling [3]. Therefore we now addressed the role of G-proteins for IR autophosphorylation in the absence of $MnCl_2$.

MATERIALS AND METHODS

Materials

Recombinant α-subunits of G_i2, Go, Gs and G$\beta\gamma$ subunits from bovine brain, the A-protomer of PTX (pertussis toxin) and anti-$G_i3\alpha$ antibody were from Calbiochem–Novabiochem (Nottingham, U.K.). Polyclonal antibodies raised against internal sequences of IRβ, $G_i1\alpha$ and $G_i2\alpha$ subunits and polyclonal antibodies against C-terminal sequences of IRβ subunits (used for Western blotting), Gαq and Gβ2 subunits were from Santa Cruz Biotechnology. Insulin, GTPγS, GDPβS (guanosine 5′-[β-thio]disphosphate) and GTP were from Roche; all other chemicals were from Sigma.

Cell culture, preparation of cells and plasma membranes

Human fat cells were isolated from non-diabetic subjects undergoing elective abdominal or cosmetic breast surgery as described previously [11,12].

Abbreviations used: GDPβS, guanosine 5′-[β-thio]disphosphate; GTPγS, guanosine 5′-[γ-thio]triphosphate; IGF-1, insulin-like growth factor 1; IR, insulin receptor; KDR, vascular endothelial growth factor receptor 2; PTX, pertussis toxin; RACK1, receptors for activated C-kinase 1.

[1] To whom correspondence should be addressed (e-mail heidemarie_krieger-brauerH@med.uni-heidelberg.de).

Immunoaffinity chromatography

IRs were purified by immunoaffinity chromatography as described in [3]. In brief, the immunoaffinity column (1.5 ml) was prepared using CNBr-activated Sepharose (Amersham Biosciences), which bound to a polyclonal antibody recognizing an amino acid sequence (1365–1382) within the intracellular epitope of the IRβ subunit (Santa Cruz Biotechnology). Plasma membranes (500 μg) preincubated with and without insulin or with PTX for 10 and 45 min respectively were collected by centrifugation and solubilized at 4 °C in 30 mM Hepes (pH 7.6), containing 4 mM EDTA, 4 mM EGTA, 1 % Triton X-100, 1 mM phenylmethylfluoride, 2 mM pepstatin and 2 μg/ml aprotinin. The proteins were subjected to the immunoaffinity matrix prewashed with 25 mM Hepes (pH 7.6), 0.1 % Triton X-100, and the protease inhibitors used for solubilization. After 12–14 h, the column was washed three times with 10 column volumes of the same buffer. Receptors and associated proteins were eluted with 50 mM $NaCO_3$ (pH 10.5), containing 0.1 % Triton X-100 and protease inhibitors. Proteins were concentrated and reconstituted in 25 mM Hepes buffer (pH 7.6), containing 0.1 % Triton X-100, 120 mM NaCl, 2.5 mM $MgCl_2$ and protease inhibitors. The protein content of the eluates was measured, and receptors were quantified by immunoblotting with anti-IRβ antibodies.

PTX treatment

Membranes (100 or 500 μg) were incubated for 45 min at 37 °C in 0.1 or 0.5 ml of 30 mM Tris/HCl (pH 7.5), 10 mM thymidine, 10 mM arginine, 2.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM ATP, 0.5 mM GTP and 2 μg/ml PTX A-protomer. ADP ribosylation was initiated by the addition of 10 μM [^{32}P]NAD (10 μCi/assay; NEN, Dupont, Melbourne, Australia). For assessing the inhibition of PTX labelling by $MnCl_2$, NaF or vanadate, membranes were first treated with 3 mM $MnCl_2$, 5 mM NaF or 1 mM vanadate for 30 min at 23 °C and then incubated with the ADP-ribosylation reaction mixture. Reactions were terminated by centrifugation, and the pellets were solubilized with 150 μl of Laemmli buffer [13]. Proteins were resolved by SDS/PAGE (10 % gel) and transferred on to Hybond PVDF membranes and visualized by autoradiography. The same membranes were probed with $G_i2\alpha$ antisera and proteins were visualized with a peroxidase-conjugated secondary antibody and the ECL® system. The conditions used for assessing the effect of PTX on IR autophosphorylation were identical with those described above, except that unlabelled NAD was added. Controls contained all reagents except the bacterial toxin. After 45 min, membranes were pelleted and washed with 30 mM Hepes (pH 7.5), containing 120 mM NaCl, 1.4 mM $CaCl_2$ and 2.5 mM $MgCl_2$ and then solubilized after immunoaffinity chromatography as described above.

In vitro IR autophosphorylation

Reaction mixtures with equal amounts of IR were incubated in 30 mM Hepes buffer (pH 7.5), containing 120 mM NaCl, 1.4 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05 % Triton X-100 in the presence of insulin and GTPγS (concentration as indicated). Phosphorylation was initiated by adding 0.5 mM ATP. After 10 min, at room temperature (23 °C), the reaction was stopped by the addition of Laemmli sample buffer. After separation by SDS/PAGE (10 % gel), proteins were transferred on to PVDF membranes blotted with 5 % polyvinylpyrrolidone, and incubated with an anti-phosphotyrosine antibody (Py99; Santa Cruz Biotechnology) and reblotted with an anti-IRβ antibody.

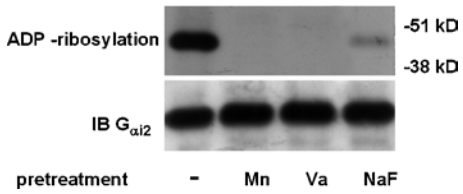

Figure 1 PTX-catalysed ADP ribosylation of G_i is prevented by $MnCl_2$, vanadate and NaF

Plasma membranes from human adipocytes were incubated in the absence (control) or presence of 3 mM $MnCl_2$, 1 mM vanadate (Va) or 10 mM NaF at 23 °C. After 30 min, PTX A-protomer (2 μg/ml) was added and incubated as described in the Materials and methods section and subjected to SDS/PAGE, followed by subsequent autoradiography and immunoblotting (IB) with anti-$G_{\alpha i2}$.

RESULTS

The effects of guanine nucleotides on the receptor tyrosine kinase

A role for G-proteins on IR catalytic activity was investigated using partially purified IRs from membranes of human adipocytes. It has been described that phosphotyrosine phosphatase inhibitors, such as NaF or vanadate are activators of G-proteins [16–19]. Also, in the present study, we confirmed this observation as evidenced by inhibition of PTX-induced ADP ribosylation of $G_i2\alpha$ (Figure 1). Therefore, in subsequent experiments, we isolated the IR in the absence of NaF and vanadate by affinity chromatography using antibodies directed against the amino acid sequence 1365–1382 of the IR β-subunit. The IR preparations were subjected to a phosphorylation assay, containing 2 mM $MgCl_2$ but no $MnCl_2$, since we demonstrated previously that $MnCl_2$ mimics the effect of GTPγS on insulin-induced activation of G_i-proteins [3]. Under these conditions, the IRs were phosphorylated already after 5 min treatment with insulin (10 nM) and GTPγS (10 μM) in the presence of 0.5 mM ATP. After 10 min, autophosphorylation of the IR was 30–40-fold over basal phosphorylation (Figure 2A).

To test the involvement of G-proteins in IR autophosphorylation, the receptor preparations were incubated with increasing concentrations of GTPγS in the presence of 10 nM insulin. GTPγS caused a concentration-dependent augmentation of insulin-induced autophosphorylation with a 3–4-fold maximum at 10 μM (Figure 2B). In the absence of insulin, GTPγS-induced autophosphorylation was 5-fold over baseline at 10–100 μM (Figure 2C). The level of insulin-stimulated IR phosphorylation was 10 ± 3-fold over baseline, and the addition of GTPγS (10 μM) increased the maximal stimulation by insulin over basal to 33 ± 6-fold (Figure 2C). Figure 2(D) shows dose–response curves for insulin in the absence and presence of maximal concentration of GTPγS (10 μM). A significant increase in autophosphorylation of IRs was observed at 100 pM insulin and the maximal effect occurred with 1 nM insulin in the presence of 10 μM GTPγS, indicating that insulin acted via its cognate receptor. When higher concentrations of GDPβS were added, insulin (10 nM)-stimulated autophosphorylation was inhibited by more than 50 % at 100 μM GDPβS (Figure 2E).

Modulation of G-protein–receptor interaction

Previously, we have shown that IRs associate with G_i2-proteins [3]. However, no regulation of this interaction was demonstrated. In the present study, we examined a ligand-induced modulation of $G_i2\alpha$ association with the IR. Hence, plasma membranes were incubated with increasing concentrations of insulin in the absence

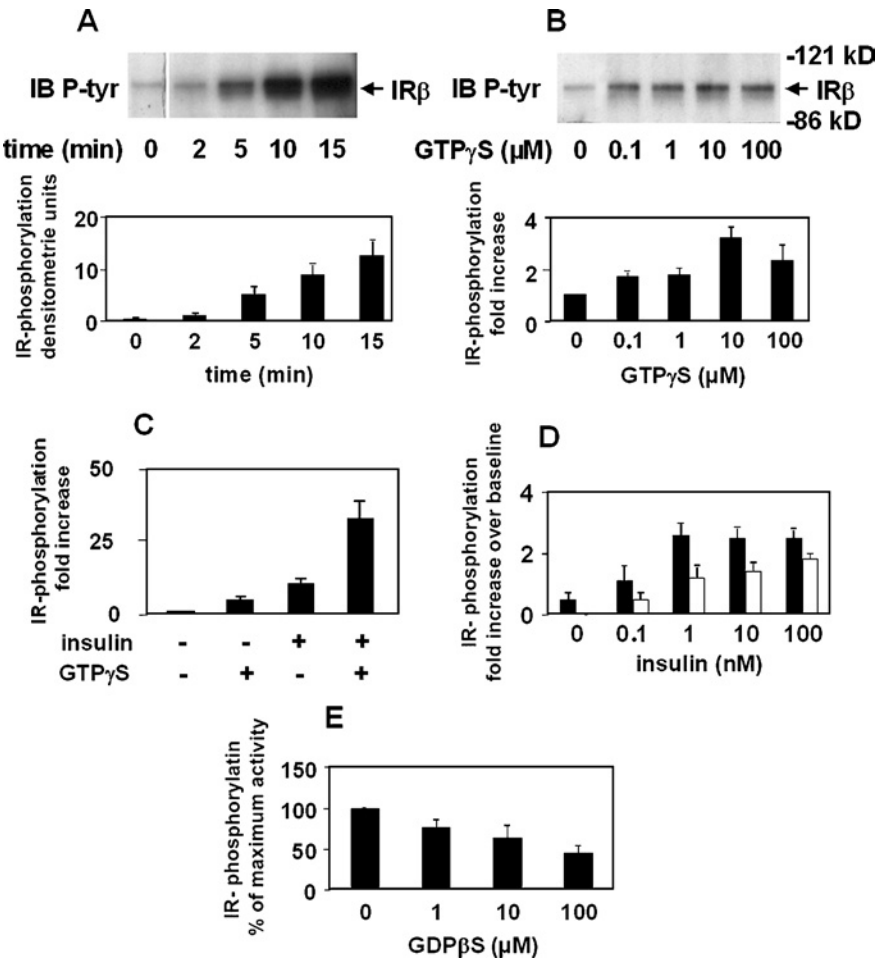

Figure 2 Effect of GTPγS and GDPβS on IR autophosphorylation

(**A**) Immunoaffinity-purified IRs were treated with insulin (10 nM) and GTPγS (10 μM) in the presence of 0.5 mM ATP for various time intervals at 23 °C. After SDS/PAGE and immunoblotting with anti-phosphotyrosine antibodies (upper panel, representative immunoblot), densitometric analysis (lower panel) of the bands was performed. Results are expressed as means ± S.D., $n = 3$. (**B**) Purified IRs were treated with increasing concentrations of GTPγS in the presence of 10 nM insulin and were incubated with 0.5 mM ATP for 10 min at 23 °C. After SDS/PAGE and immunoblotting with anti-phosphotyrosine and anti-IRβ (upper panel), densitometry was performed (lower panel). Results are normalized to the amount of IRβ and presented as means ± S.D. fold increase in phosphorylation as compared with insulin stimulation alone, $n = 3$. (**C**) Purified IRs were incubated with and without GTPγS (10 μM) or insulin (10 nM) in the presence of 0.5 mM ATP for 10 min. Results are expressed as means ± S.D. fold increase in phosphorylation as compared with baseline values in the absence of GTPγS and insulin, $n = 6$. (**D**) Immunoaffinity-purified IRs were treated with increasing concentrations of insulin in the absence (white bars) or presence (black bars) of 10 μM GTPγS and allowed to autophosphorylate in the presence of 0.5 mM ATP for 10 min. After SDS/PAGE and immunoblotting with anti-phosphotyrosine and anti-IRβ, densitometry was performed. Results are normalized to the amount of IRβ and presented as means ± S.D. fold increase in phosphorylation as compared with baseline in the absence of insulin and GTPγS, $n = 3$. (**E**) Purified IRs were incubated with 10 nM insulin and 0.5 mM ATP in the presence of increasing concentrations of GDPβS for 10 min. Results are expressed as percentage of insulin-stimulated phosphorylation, $n = 3$.

of GTPγS and ATP. The receptor–G-protein complex was then isolated by immunoaffinity chromatography and the column eluates were assayed for IR, $G_i1\alpha$, $G_i2\alpha$, $G_i3\alpha$, Gαq and Gβ subunits by immunoblotting (Figures 3A and 3B). In the absence of insulin, the IR was co-purified with its associated heterotrimeric G-protein complex. Thus the IR fractions yielded positive immunoreactivity for $G_i2\alpha$, Gαq and Gβ2 subunits. No reactions were found with antibodies directed against $G_i1\alpha$ and $G_i3\alpha$ (Figure 3B). Insulin treatment induced an increase in $G_i2\alpha$ and Gβ2 association with the IR (Figures 3A and 3C). Insulin treatment produced no change in the amounts of Gαq subunits (Figure 3A), consistent with recent observations by others [15,18].

Modulation of receptor phosphorylation by G_i

We then investigated functional consequences of an increased G_i2 association with the IR on the autophosphorylation levels. The IR fractions isolated from untreated and insulin-treated membranes were assayed for receptor kinase activity. We found that the extent of IR phosphorylation was proportional to the amount of associated G_i2-proteins caused by insulin treatment (Figures 3C and 3D). These results indicate that increased associated G_i2-proteins with IR increase the ability of insulin to activate receptor autophosphorylation, suggesting that G_i2-proteins are involved in IR autophosphorylation.

Effect of PTX

Several years ago it was reported that treatment of intact cells with PTX decreased IR kinase activity, but this effect of PTX could not be demonstrated under *in vitro* conditions [19]. Now we have isolated IRs from membranes pretreated with the A-protomers of PTX. The effects of PTX on the IR kinase activity are shown in Figure 4(A). IR autophosphorylation stimulated by insulin and

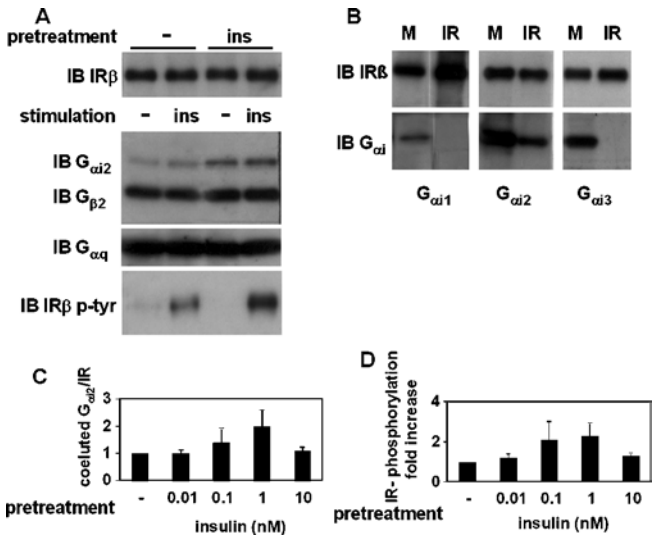

Figure 3 Binding of G_i to the IR

(**A**) Insulin-stimulated association of $G_{\alpha i2}$ and $G_{\beta 2}$ with the IR. Plasma membranes were incubated with increasing concentrations of insulin for 5 min, solubilized and subjected to anti-IR immunoaffinity chromatography. After washing, proteins were eluted and subjected to SDS/PAGE and immunoblotted with anti-IRβ, anti-$G_{\alpha i2}$, anti-$G_{\alpha q}$ or anti-$G_{\beta 2}$. Representative immunoblots, $n = 3$. (**B**) Association of G_i-proteins with the IR. Solubilized membranes (M) or purified IR were subjected to SDS/PAGE and immunoblotted with anti-IRβ, anti-$G_{\alpha i1}$, anti-$G_{\alpha i2}$ or anti-$G_{\alpha i3}$ antibodies. Representative immunoblots out of three separate experiments. (**C**) Insulin-stimulated association of $G_{\alpha i2}$ and $G_{\beta 2}$ with the IR, densitometric analysis of the blots described in (**A**). Results are expressed as means $\pm$ S.D. and were normalized to the amount of IR. (**D**) Effect of increasing G_{i2}-association with the IR on autophosphorylation. Insulin-pretreated immunoaffinity-purified IRs from (**C**) were incubated with 10 nM insulin and 10 μM GTPγS in the presence of 0.5 mM ATP for 10 min, subjected to SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies. The blots from three different experiments were quantified by densitometry. Results are normalized to the amount of IR and presented as means $\pm$ S.D. fold increase in phosphorylation as compared with values of IR phosphorylation from untreated membranes.

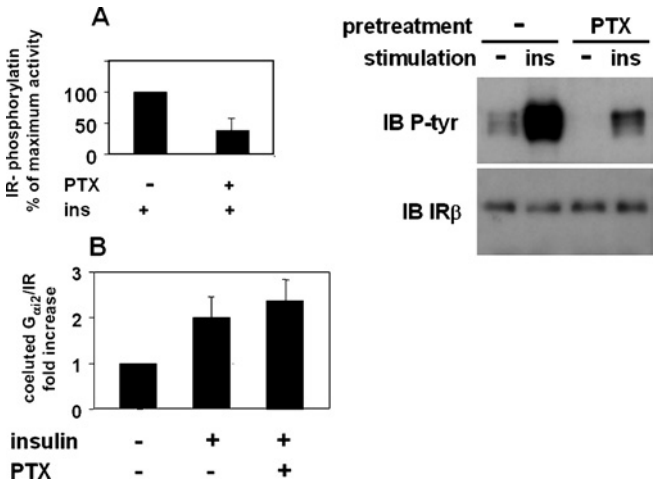

Figure 4 Effect of PTX

(**A**) Effect of PTX on autophosphorylation of the IR. Plasma membranes from human adipocytes were pretreated in the absence or in the presence of 2 μg/ml PTX A-protomer (PTX) for 45 min as described in the Material and methods section. After washing, membranes were solubilized and subjected to anti-IR immunoaffinity column. Purified IRs from control and PTX-treated membranes were stimulated with 10 nM insulin, 0.5 mM ATP and 10 μM GTPγS for 10 min. After SDS/PAGE and immunoblotting with anti-phosphotyrosine and anti-IRβ, densitometry of the bands was performed. Results are normalized to the amount of IRβ and are expressed as percentage of IR phosphorylation from membranes without PTX pretreatment, $n = 6$. Right panel: a representative immunoblot. (**B**) Effect of PTX on G_i association with the IR. Plasma membranes pretreated with and without PTX A-protomer (2 μg/ml) were incubated with 1 nM insulin for 5 min. After solubilization, IRs were purified by immunoaffinity chromatography, subjected to SDS/PAGE and immunoblotted with anti-IRβ and anti-$G_{\alpha i2}$. Results are normalized to the amount of IRβ and are means $\pm$ S.D., $n = 6$.

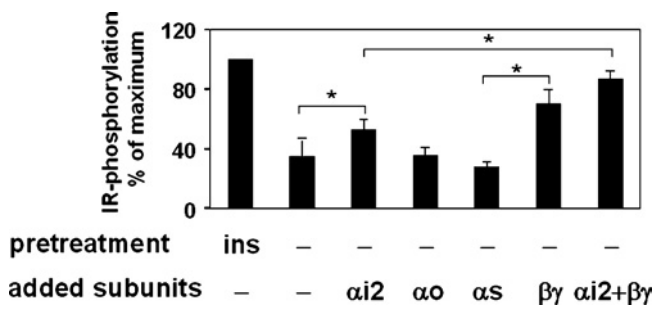

Figure 5 Effect of recombinant Gα subunits and isolated G$\beta\gamma$ subunits on IR autophosphorylation

Purified IRs from untreated adipocyte membranes were incubated with or without nucleotide-linked recombinant Gα subunits (100 nM), or with G$\beta\gamma$ subunits (200 nM) in the presence of 10 μM GTPγS, 0.5 mM ATP and 1 nM insulin for 10 min to allow receptor autophosphorylation. Purified IRs from membranes pretreated with insulin (ins, 1 nM), which were then incubated for 10 min in the presence of 10 μM GTPγS, 0.5 mM ATP and 1 nM insulin, served as a positive control. After SDS/PAGE and immunoblotting with anti-phosphotyrosine and anti-IRβ, the blots were quantified by densitometry. Results (means $\pm$ S.D.) from six independent experiments are normalized to the amount of IRβ and expressed as percentage of phosphorylation of IRs isolated from insulin-pretreated membranes (positive control). $^*P < 0.05$.

GTPγS was inhibited by PTX pretreatment. To determine whether the functional uncoupling of G_i-proteins results from the inhibition of the interaction of G_i-proteins with the IR, the effect of PTX on IR–G_i2 association was evaluated in IR fractions from membranes treated with PTX A-protomer in the absence or presence of insulin. As shown in Figure 4(B), the interaction of G_i2 with the IR was enhanced by insulin treatment, and this interaction was not disrupted by PTX pretreatment. A similar observation was reported for isolated IGF-1 (insulin-like growth factor 1) receptors and associated G_i-proteins [20]. However, this is not a general feature of PTK receptor–G-protein interaction but is specific for the IR, since the interaction of G_i2-proteins with platelet-derived growth factor α-receptors was disrupted by PTX as we have shown previously [21]. Our results strongly suggest that ADP ribosylation of the C-terminus of $G_i2\alpha$ subunits does not disrupt the IR–G_i2 complex but hampers the ability of insulin to stimulate autophosphorylation of the IR.

Regulation of IR phosphorylation by recombinant Gα and G$\beta\gamma$ subunits

To confirm further the involvement of $G_i2\alpha$ or G$\beta\gamma$ subunits in regulating receptor kinase activity, we examined the ability of recombinant G-protein subunits to induce autophosphorylation of the IR. The activation of Gα subunits and detection of functional G-protein heterotrimers by ADP ribosylation were performed as described elsewhere [3]. The addition of GTPγS-activated $G_i2\alpha$ subunits to IR fractions from untreated membranes caused an increase in insulin-induced autophosphorylation, whereas GTPγS-activated Gαo subunits were without effect (Figure 5). In contrast, addition of Gαs decreased the autophosphorylation induced by insulin. Addition of G$\beta\gamma$ subunits also resulted in an increase in autophosphorylation. The combination of both $G_i2\alpha$ and G$\beta\gamma$

subunits did further enhance insulin-dependent phosphorylation. These results indicate that IR autophosphorylation can be activated by both subunits of G_i2-proteins.

DISCUSSION

The present study describes a possible involvement of G_i-proteins in the regulation of IR kinase activity. Four lines of evidence are provided for a G-protein-dependent regulation of IR tyrosine kinase activity: (i) The G-protein-activator GTPγS stimulated not only autophosphorylation of the IR in the absence of insulin but also the insulin-induced autophosphorylation was 3-fold enhanced by the addition of GTPγS. (ii) IR autophosphorylation induced by insulin was inhibited by GDPβS, an inhibitor of G-protein activation. (iii) Stimulatory effects of insulin and GTPγS were prevented in receptor preparations isolated from PTX-pretreated membranes, consistent with an earlier report of Müller-Wieland et al. [19], who showed the inhibitory effect of PTX on IR autophosphorylation in intact FaO cells. (iv) The addition of GTPγS-activated $G_i2\alpha$ or G$\beta\gamma$ subunits to IR preparations enhanced insulin-induced autophosphorylation.

Currently, no definitive insights into the role of G-proteins in regulating IR kinase activity have been provided. Previous studies failed to demonstrate the inhibition of receptor tyrosine kinase activity by PTX or GDβS [7–9,19]. *In vitro* studies on the IR tyrosine kinase activity are generally performed in the presence of millimolar concentrations of Mn^{2+}, because this cation is considered to be essential for ligand-induced activation of tyrosine kinase activity in cell-free preparations. Furthermore, the phosphatase inhibitors, vanadate and NaF, are generally present in receptor preparations. All three components activate heterotrimeric G-proteins, thereby preventing PTX-induced ADP ribosylation of $G_{i/o}$-proteins [3,14–17,22]. Thus one of the main obstacles in elucidating the role of G-proteins in regulating receptor kinase may reside in the fact that the reported experimental conditions were inappropriate for investigating G-protein action in cell-free IR preparations.

The present and earlier studies indicate that the IR is coupled with G_i2 [3,4]. The IR, similar to the receptor for platelet-derived growth factor AA, activates membrane-bound NADPH oxidase via $G_i\alpha2$-proteins, independent of receptor kinase activity [3,21]. This leads to the generation of reactive oxygen species in a variety of cells stimulated with cytokines, growth factors and agonists of seven-helix receptors. This in turn may mediate specific cellular functions such as cell growth, differentiation and apoptosis. G_i2-proteins and the reactive oxygen species also play an important role in the differentiation of adipocytes induced by insulin [23,24]. In addition, constitutive activation of $G_i2\alpha$ in transgenic mice is insulinomimetic with regard to glucose transport and mitogen-activated protein kinase activation [25,26]. In contrast, $G_i2\alpha$ deficiency has been shown to provoke insulin resistance [27]. Involvement of G_i2 in regulating IR kinase activity may explain the essential role of G_i2 in insulin action. Except for NADPH oxidase, effectors directly regulated by G_i2 in insulin signalling are not known. Our findings support and extend earlier assumptions and suggest that the IR itself can be an effector system of G_i2. Furthermore, the IRs, utilizing G_i2- and Gq-proteins, can modulate other effectors such as the NADPH oxidase or glucose transport.

Recent reports indicate that non-receptor tyrosine kinase activation is mediated by Gα and G$\beta\gamma$ subunits of heterotrimeric G-proteins. Bruton's tyrosine kinase is stimulated by G$\beta\gamma$ via interaction with PH domains (pleckstrin homology domains) and by Gαq and G12α through binding on PH/Bruton's tyrosine kinase motif domains of the kinase [28–30]. Recently, it has been reported that activated forms of Gα-proteins, $G_i\alpha$ and $G_s\alpha$, directly interact with and thereby activate Src kinase via the catalytic domain of the kinase [31].

The mechanism of heterotrimeric G-protein-dependent stimulation of IR kinase activity is unknown. One mechanism may be that G_i2-proteins activate the receptor kinase through direct interaction. Addition of activated $G_i2\alpha$ and G$\beta\gamma$ subunits activates the receptor kinase only in the presence of insulin. This suggests that the ligand-dependent conformational change may be necessary to induce interaction of intracellular signalling molecules [32]. A recent report describes the interaction of the vascular endothelial growth factor receptor 2 (KDR) with Gq/11 independent of KDR phosphorylation. Gq/11 mediated KDR phosphorylation, which is comparable to our observation with G_i and the IR [33]. Another possibility may be the existence of a G-protein regulator protein (RGS protein) that mediates IR-induced G_i2 activation. GAIP (GTPase-activating protein for G_i) and GIPC (GAIP-interacting protein, C-terminus) have been shown to be directly associated with tyrosine kinase receptors for IGF-1 and NGF (nerve growth factor). This physical interaction between regulator proteins and tyrosine kinase receptors may mediate tyrosine kinase receptor and G-protein signalling [34,35]. The differential effect of $G_i2\alpha$ and G$\beta\gamma$ subunits on IR phosphorylation probably indicate an involvement of RGS proteins. These proteins inhibit G-protein signalling at the level of G-protein α-subunits by accelerating the rate of intrinsic GTPase reaction or by competing with effectors for Gα binding and, therefore, could play a controlling role in the effectiveness of receptor ligands [36,37].

Negative regulation of IR kinase by dephosphorylation through tyrosine phosphatases, including PTP (protein tyrosine phosphatase) 1B, LAR (leucocyte common antigen-related molecule) and PTPα is well documented [38–40]. It has been shown that the insulin-induced binding of the adapter protein, Grb14, on the phosphorylated tyrosine kinase loop inhibits the catalytic activity of the kinase *in vitro* [41]. Another interacting protein is RACK1 (receptors for activated C-kinase 1), which has only recently been identified. RACK1 associates with insulin and IGF-1 receptors in a tyrosine-kinase-independent manner and IGF-1 receptor kinase activity is enhanced in receptor preparations from RACK1-overexpressing cells [42]. RACKs are a family of proteins that share homology with Gβ subunits. RACK1 is considered as a scaffold protein that interacts with several proteins. Interestingly, RACK1 has also been found to interact with heterotrimeric G-proteins, a surprising result because very few proteins, other than receptors, interact with the whole heterotrimer [43]. Further investigations will be required to elucidate the mechanism by which the IR activates G_i2-proteins, and by which IR kinase is regulated by G_i2.

We thank B. Sattel and K. Hanna for expert technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn, Germany. This paper is dedicated to our former colleague Professor Dr H. Kather, who passed away in 2001.

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Received 30 October 2003/18 February 2004; accepted 16 March 2004
Published as BJ Immediate Publication 16 March 2004, DOI 10.1042/BJ20031659