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Research Article

Insulin activates $G\alpha_{il,2}$ protein in rat hepatoma (HTC) cell membranes

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Abstract. Insulin action is initiated by binding to its cognate receptor, which then triggers multiple cellular responses by activating different signaling pathways. There is evidence that insulin receptor signaling may involve G protein activation in different target cells. We have studied the activation of G proteins in rat hepatoma (HTC) cells. We found that insulin stimulated binding of guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-^{35}S) to plasma membrane proteins of HTC cells, in a dose-dependent manner. This effect was completely blocked by pertussis toxin treatment of the membranes, suggesting the involvement of G proteins of the $G\alpha_i$/$G\alpha_o$ family. The expression of these $G\alpha$ proteins was checked by Western blotting. Next, we used blocking antibodies to sort out the specific $G\alpha$ protein activated by insulin stimulation. Anti-$G\alpha_{i1,2}$ antibodies completely prevented insulin-stimulated GTP binding, whereas anti-$G\alpha_{o,i3}$ did not modify this effect of insulin on GTP binding. Moreover, we found physical association of the insulin receptor with $G\alpha_{i1,2}$ by copurification studies. These results further support the involvement of a pertussis toxin-sensitive G protein in insulin receptor signaling and provides some evidence of specific association and activation of $G\alpha_{i1,2}$ protein by insulin. These findings suggest that $G\alpha_{i1,2}$ proteins might be involved in insulin action.

Key words. Insulin; insulin receptor; G protein; insulin action; cellular signaling; hepatocytes.

The interaction of insulin with target cells is mediated by a specific $\alpha_2\beta_2$ glycoprotein receptor in the plasma membrane [1–4]. Insulin binds to the extracellular α-subunit and then stimulates intracellular β-subunit tyrosine kinase activity. This tyrosine kinase activity is one of the earliest steps in insulin action and is essential for many biological effects of insulin [5]. However, there is also evidence indicating that tyrosine phosphorylation may not be essential in all cases for insulin receptor signaling [6–9] and insulin action [10–15]. One of the proposed pathways independent of tyrosine phosphorylation may be via G proteins [9, 16–18]. In this line, it has been shown that the insulin receptor may interact with pertussis toxin-sensitive G proteins (i.e. members of the $G\alpha_i$/$G\alpha_o$ family) [19–23]. Moreover, GTP-binding protein-activator sequences have been found in the insulin receptor [24]. Regarding the physiological relevance of this signaling pathway of the insulin receptor, downregulation of G_i subtypes of G proteins has been associated with in vitro insulin resistance [25], and altered cross-talk between the insulin receptor and G_i proteins has been found in obese type 2 diabetes [26]. These data suggest a direct interaction of the insulin receptor with G proteins of the G_i family. In the present work we studied the cross-talk of insulin receptors with G proteins by testing hormone-dependent guanosine 5′-*O*′-(3-thiothriphosphate) (GTP-γ-S) binding to HTC membranes. The specific G protein subtype coupled to

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the insulin receptor was assessed by using Gα protein antibodies, and the specific association was checked by copurification studies.

Material and methods

Materials. Protein A coupled to Sepharose 4B and WGA coupled to agarose were from Pharmacia Biotech (Uppsala, Sweden). Bacitracin, leupeptin, PMSF (phenylmethanesulphonyl fluoride), TLCK (*N*-*p*-tosyl-L-lysine chloromethyl ketone), pepstatin, aprotinin, dithiothreitol (DTT), *N*-acetyl-β-glucosamine (NAG), and bovine serum albumin (BSA) (fraction V) were from Sigma (St. Louis, MO, USA). GTP, GDP (guanosine/diphosphate), GTP-γ-S and other nucleotides were from Boehringer Mannheim (Barcelona, Spain). Electrophoretic chemicals and molecular weight standards were from Novex (San Diego, CA, USA). Rabbit antisera against $\alpha_{i1,2}$-, $\alpha_{o,i3}$- and β_{common}-subunits of G proteins were from Du Pont NEN (E. I. du Pont de Nemours, Germany). GTP-γ-^{35}S (1000 Ci/mmol) was from Amersham Iberica (Madrid, Spain).

Cells and preparation of membranes. Rat HTCs were kindly provided by Dr. Ira D. Goldfine (UCSF, San Francisco, CA, USA). Cells were prepared and maintained in Dulbecco's modified Eagle's medium (DMEM) as previously described [27, 28]. For membrane preparation, cells were washed and harvested in phosphate-buffered saline (pH 7.4). The cell suspension was next centrifuged at 4 °C (300*g*) for 10 min. The supernatant was discarded and the pellet resuspended in homogenation buffer: 20 mM Hepes (pH 7.4) supplemented with bacitracin (200 μg/ml), PMSF (0.1 mM), TLCK (10 μg/ml), leupeptin (10 μg/ml) pepstatin (5 μg/ml) and aprotinin (10 μg/ml). Cells were homogenized and the homogenate was centrifuged at 4 °C (1000*g*) for 10 min. The supernatant was then centrifuged at 4 °C (45,000*g*) for 30 min, the pellet was washed once with the same buffer and repelleted to collect crude plasma membranes, which were stored at − 80 °C.

Pertussis toxin pretreatment of membranes. The treatment of membranes with thiol-preactivated pertussis toxin was carried out as previously described [29]. Briefly, membranes were incubated with thiol-preactivated pertussis toxin (1 μg/ml) for 1 h at 23 °C in 25 mM Hepes buffer (pH 7.4) containing 5 mM $MgCl_2$, 10 mM NAD^+, 1 mM adenosine triphosphate (ATP), 2 mM creatin phosphate, 0.1 mg/ml creatin kinase and 0.1 mM GTP. Control membranes were treated in an identical manner, except that there was no toxin present in the incubation. The membrane suspension was then centrifuged and the pellet washed twice and finally resuspended in Hepes buffer (20 mM, pH 7.5), containing bacitracin (200 μg/ml) and PMSF (0.1 mM).

GTP-γ-^{35}S binding assay. GTP binding assay was conducted at 23 °C in a buffer consisting 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10^{-4} M GDP and 10 mM Tris, pH 7.5, containing 0.5 nM GTP-γ-^{35}S (0.1 μCi per assay tube) [30]. The reaction was started by the addition of HTC membranes (100 μg), and stopped at different times by adding 1 ml of cold buffer. After centrifugation at 15,000*g* at 4 °C and two washes with cold buffer, the pellet was dissolved in a scintillation cocktail and counted in a scintillation counter (Wallac 1409, Turku, Finland). Nonspecific binding was determined in the presence of 10^{-5} M cold GTP-γ-S. All assays were performed in quadruplicate.

Blocking experiments with antisera were performed by the addition of the specific antisera (1:100 final dilution) to the plasma membranes and incubation for 2 h at 4 °C with gentle agitation as previously described [31–33]. The 1:100 dilution of sera was found to be the dilution with maximum blocking effect as observed in previous work [31–33].

Immunodetection of insulin receptor β subunit, $\alpha_{i1,2}$, $\alpha_{o,i3}$ and β subunits of GTP-binding proteins. Membranes, semipurified and purified receptors were denatured with Laemmli buffer and run on polyacrylamide gel electrophoresis (SDS-PAGE) (8–16%) [34]. Proteins were electrophoretically transferred onto nitrocellulose membranes. After blocking with 5% skimmed milk, the membranes were first incubated with anti-IRβ, anti-β_{common}, anti-$\alpha_{o,i3}$ or anti-$\alpha_{i1,2}$ washed three times, further incubated with the second antibody conjugated with horseradish peroxidase and developed by the Amersham enhanced chemiluminescence (ECL) detection system [35].

Insulin receptor purification. Insulin receptors were semipurified by wheat germ agglutinin chromatography from HTC cells as previously described [6, 36]. Cells were solubilized in 1% Triton X-100 containing 50 mM Hepes, pH 7.6, 1 mM PMSF, 1 mM sodium orthovanadate and 0.1 mg/ml aprotinin. Next, the solubilized cells were centrifuged at 50,000*g* for 1 h. The soluble extract was purified on a 2-ml column containing wheat germ agglutinin coupled to agarose and eluted with 0.3 M *N*-acetyl-β-glucosamine as previously described [37].

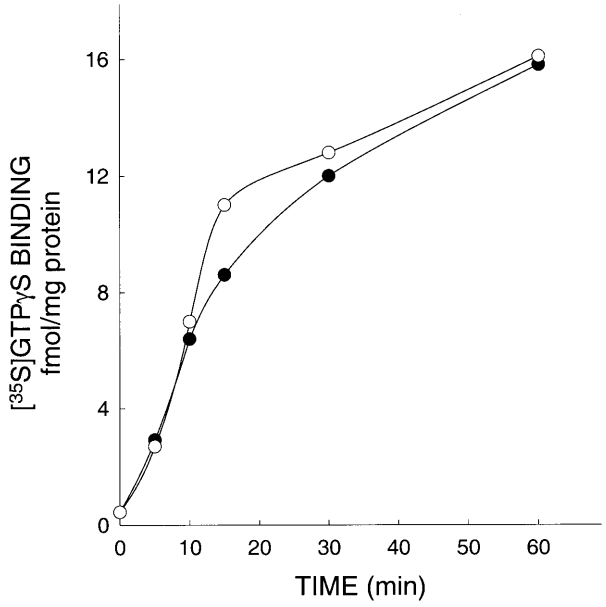

Figure 1. Effect of insulin on GTP-γ-S binding to HTC cell membranes. HTC cell membranes were incubated with γ-^{35}S-GTP in the absence (●) or presence (○) of insulin (10^{-8} M), and the binding was determined as described under 'Materials and methods' at different time points. An experiment representative of three is shown.

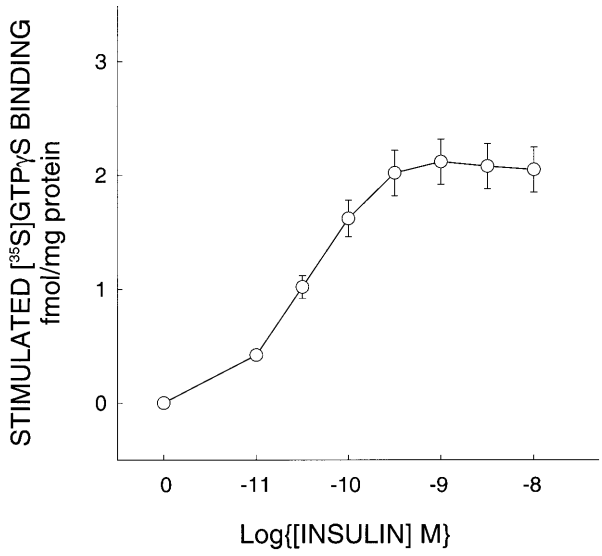

Figure 2. Concentration dependency of insulin-stimulated GTP-γ-S binding to HTC cell membranes. Membranes were incubated at 23 °C for 15 min in the presence of increasing concentrations of insulin to determine GTP binding activity as described under 'Materials and methods'. The values of each experiment were based on quadruplicate tubes. Data are means ($n = 4$) of the increase in GTP-binding activity above control levels.

Semipurified material was further purified by immunoaffinity with anti-insulin receptor antibodies coupled to Protein A-Sepharose. Insulin receptors were immunoprecipitated as previously described [36, 38]. Immunoprecipitates were washed and denatured in SDS sample buffer to be analyzed by SDS-PAGE and Western blotting [27, 36].

Results

Insulin stimulation of GTP-γ-S binding. In receptor systems that are coupled to G proteins, ligand binding increases GTP binding to the α subunit of the G proteins [39]. Therefore, the effect of insulin on GTP-γ-S was analyzed in HTC membranes. Figure 1 shows the association kinetics of GTP-γ-S binding to HTC cell membranes. Specific binding reached equilibrium at 30 min. Insulin stimulated the rate of association, reaching equilibrium binding at 15 min (fig. 1). To determine the dose-response manner of the effect of insulin on the binding of GTP-γ-S, membranes were incubated with increasing concentrations of insulin for 15 min. As shown in figure 2, insulin effect was detectable at 10^{-11} M and reached its maximum at 10^{-9} M. This dose-response effect of insulin on GTP-γ-S binding showed similar characteristics to those of other insulin effects previously described in HTC cells, such as aminoisobutiric acid uptake [10].

When HTC cell membranes were pretreated with pertussis toxin, this effect of insulin on GTP-γ-S binding was completely blocked (table 1). This result suggests that insulin receptor may be coupled to pertussis toxin-sensitive G proteins and therefore belonging to the $G\alpha_i/G\alpha_o$ family.

In order to sort out the specific G protein subtype coupled to the insulin receptor, we employed blocking

Table 1. Effect of pertussis toxin pretreatment of HTC cell membranes on insulin-stimulated GTP-γ-S binding. Membranes were first preincubated with or without pertussis toxin (PT) as described under 'Materials and methods'. Thereafter, GTP-γ-S binding was measured with or without insulin (10 nM). Data are means ± SEM ($n = 3$).

	GTP-γ-S binding (fmol/mg protein)	
	Control membranes	PT pretreated membranes
Basal	8.9 ± 0.4	8.5 ± 0.5
Insulin	11.2 ± 0.3	9.1 ± 0.5*

*$P < 0.05$ versus control membranes (Student's t-test).

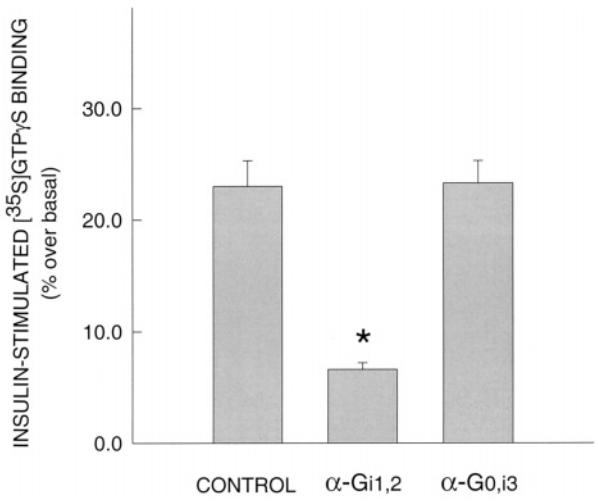

Figure 3. Effect of blocking anti-G protein antibodies on insulin-stimulated GTP-γ-S binding to HTC cell membranes. Insulin-stimulated (10 nM) GTP-γ-S binding to HTC cell membranes was measured with or without preincubation for 2 h with different blocking sera: $G\alpha_{i1,2}$, $G\alpha_{o,i3}$ or preimmune sera (C, control). Data are means $\pm$ SEM of four different experiments run in quadruplicates. $^*P < 0.001$, significant differences versus control.

antibodies in the GTP-γ-S binding assay. As shown in figure 3, anti-$G\alpha_{i1,2}$ completely blocked the insulin effect on GTP binding to HTC cell membranes, whereas anti-$G\alpha_{o,i3}$ did not affect insulin-stimulated GTP binding. These results suggest that insulin receptor couples to the pertussis toxin-sensitive G protein subtype $G\alpha_{i1,2}$ in HTC cell membranes.

Association of the insulin receptor with G proteins. Since $G\alpha_{i1,2}$ protein seems to be coupled to the insulin receptor, we checked the physical association of both components. Therefore, we looked at the presence of G proteins in WGA-purified insulin receptors. As shown in figure 4, $G\alpha_{i1,2}$ as well as $G\alpha_{o,i3}$ were present in the semipurified insulin receptor preparation from HTC cells, suggesting that some type of direct or indirect association may take place. Moreover, we further purified WGA semipurified insulin receptor by anti-insulin receptor antibodies coupled to Protein A-Sepharose. Again, $G\alpha_{i1,2}$ was present in the purified material (fig. 4), along with the β-subunit of the G protein, whereas $G\alpha_{o,i3}$ did not survive immunoaffinity purification. The presence of the insulin receptor was also checked by immunoblot in the same samples. These results suggest that other receptors (glycoproteins) may account for the association of $G\alpha_{o,i3}$ in the WGA chromatography, and demonstrate the association of the insulin receptor with the heterotrimeric $G\alpha_{i1,2}$ protein. Thus, insulin receptor seems to be coupled both functionally and physically to $G\alpha_{i1,2}$ protein in HTC cell membranes to transmit the signal inside the cell.

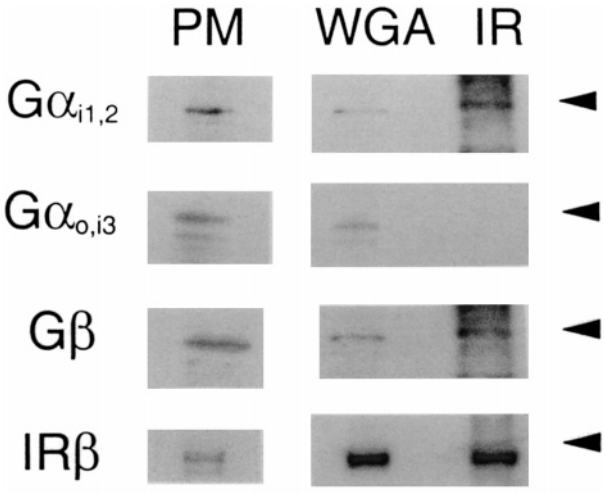

Figure 4. Association of $G\alpha_{i1,2}$ and $G\beta$ subunits with the purified insulin receptor. Membrane proteins (PM), partially purified insulin receptors by WGA (WGA) and WGA plus immunoaffinity-purified insulin receptors (IR) were subjected to SDS-PAGE and Western blotting as described under 'Materials and methods'. Blots were probed with anti-$G\alpha_{i1,2}$, anti-$G\alpha_{o,i3}$, anti-β_{common} or anti-IRβ (insulin receptor β-subunit). An experiment representative of three different purification preparations is shown.

Discussion

Association of insulin receptor with G proteins has been supported by many indirect studies in different cells and tissues. In the present study, we attempted to test the interaction between the insulin receptor and specific G protein subtypes in HTC cell membranes by two complementary approaches. Functional coupling was assessed by GTP-γ-S binding assay, and physical association was checked by copurification studies and detection by specific immunoblot.

We have found that insulin stimulates GTP-γ-S binding to HTC cell membranes, and this effect was sensitive to pertussis toxin. As previously found by others in BC3H-1 myocyte membranes [19], autophosphorylation of the insulin receptor does not seem to be necessary to exert this effect of insulin on G protein activation, since the GTP binding assay buffer did not contain ATP. These results are in agreement with previous studies in fat cells reporting pertussis toxin-sensitive insulin stimulation of GTP-γ-S binding to isolated membranes [40]. Besides, other studies have found evidence for the coupling of the insulin receptor with pertussis toxin-sensitive G proteins in rat liver membranes [16, 41] and BC3H-1

myocytes [12]. These data pointed to the participation of a G protein of the $G\alpha_i/G\alpha_o$ family. On the other hand a recent paper has shown dual coupling of the insulin receptor with both pertussis toxin- and cholera toxin-sensitive G proteins [42], suggesting that different signaling pathways may be used by insulin receptor depending on the target cell.

Nevertheless, it should be pointed out that our data regarding the kinetic of GTP binding showed that the response to insulin stimulation was slower and less efficient than that previously observed in typical G protein-coupled receptors. Even though the temperature employed (23 °C) may partially account for these results, an alternative explanation considering indirect activation of G proteins by the insulin receptor should be taken into account. We do not know yet which other proteins may have such an intermediary role, although insulin receptor substrates may be ruled out since tyrosine phosphorylation is not necessary for this effect.

On the other hand, by using specific blocking antibodies, we have found that the specific pertussis toxin-sensitive G protein subtype coupled to the insulin receptor in HTC cells is $G\alpha_{i1,2}$ rather than $G\alpha_{o,i3}$. These results agree with indirect data suggesting the role of $G\alpha_{i2}$ in insulin action, since the deficiency of this $G\alpha$ protein has been shown to impair insulin action in transgenic mice with inducible expression of RNA antisense to $G\alpha_{i2}$ [43]. Moreover, other authors have found insulin resistance in adipocytes by stimulation with agonists that downregulate G_i protein subtypes [25]. In this line, a decrease in $G\alpha_{i1,2}$ expression has been found in liver from experimentally induced type 1 diabetic rats [44], as well as in liver plasma membranes from patients with type 2 diabetes [26], suggesting a role of this pathway in the mechanism of insulin resistance. The use of anti-$G\alpha_{i1,2}$ to block insulin action has recently been used in adipocyte membranes, where this G protein subtype seems to mediate insulin stimulation of NADPH oxidase [45].

Consistent with the functional coupling of the insulin receptor with the $G\alpha_{i1,2}$ protein, we found the association of these signaling components by copurification studies. Physical association of the insulin receptor with some G proteins has previously been found in other systems such as adipocytes [46] and placenta [46, 47]. Furthermore, copurification by immunoaffinity of the insulin receptor with $G\alpha_i$ has recently been found in human adipocyte plasma membranes [45]. We have found that both $G\alpha_{i1,2}$ and $G\alpha_{o,i3}$ are present in the WGA-purified material, whereas only anti-$G\alpha_{i1,2}$ antibody was able to block the insulin-stimulated GTP binding. However $G\alpha_{o,i3}$ did not survive a further purification step by immunoaffinity chromatography on Sepharose coupled to anti-insulin receptor, suggesting that other receptors of glycoprotein nature may account for the presence of $G\alpha_{o,i3}$ in WGA-purified material. On the other hand, we have demonstrated that $G\alpha_{i1,2}$ association with insulin receptor survives not only WGA semipurification but also further immunoaffinity purification with α-insulin receptor antibodies, suggesting the physical association of the insulin receptor with $G\alpha_{i1,2}$, either directly or mediated by other unknown protein components of the signaling.

In conclusion, this study confirms the hypothesis that other pathways of insulin receptor independent of the receptor kinase exist in the cell membrane, such as the involvement of heterotrimeric G proteins. Thus, we have found that one of these pathways takes place via $G\alpha_{i1,2}$ protein in HTC cells.

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