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INSULIN STIMULATES ASSOCIATION OF A 41KDA G-PROTEIN (G_{IR41}) WITH THE INSULIN RECEPTOR

Hanjoong Jo, Stephanie Byer and Jay M. McDonald*

Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294

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SUMMARY Insulin has a paradoxical effect on a 41kDa Gi-like protein: Although insulin-treatment of rat adipocytes inhibited pertussis toxin-catalyzed ADP-ribosylation of a 41kDa G-protein in membranes in a dose-dependent manner, it simultaneously increased ADP-ribosylation of a 41kDa G-protein that co-immunoprecipitates with the insulin receptor (G_{IR41}). The latter effect was insulin concentration- and time-dependent. The dose-dependent stimulatory effect of insulin on the autophosphorylation of the insulin receptor and on the ADP-ribosylation of the GIR41 in the insulin receptor immunoprecipitates closely paralleled each other. The time course of insulin-stimulated increase in the ADP-ribosylated G_{IR41}, although rapid, was slower than the autophosphorylation of the receptor. The G_{IR41} is associated with and regulated by the insulin receptor further supporting an important role for this G-protein in modulating insulin action at the receptor level.

Substantial evidence indicates that GTP-binding proteins (G-proteins) may modulate the insulin receptor kinase activity (1-5). At least three different classes of G-proteins have been shown to be implicated in insulin receptor signalling pathway. These are 1) conventional heterotrimeric Gi- (3,4,6-9) and Gs-proteins (10,11), 2) p21ras-protein (12,13) and 3) a novel 66 to 67kDa G-protein (3,14,15). Of these G-proteins, p21ras-protein is likely to be regulated by intermediate factors such as GTPase activating protein (16) or IRS-1 signalling complexes including growth factor-bound protein 2 (GRB2) and guanine nucleotide releasing factors (17,18). The insulin receptor is associated with two G-proteins of 67 and 41kDa, (G_{IR67} and G_{IR41}) (3,14). Recently, we have provided evidence that a non-phosphorylated peptide,

* To whom correspondence should be addressed. FAX: 975-9927.

ABBREVIATIONS: G-protein, GTP-binding protein; Gi, an inhibitory G-protein; G_{IR41} or G_{IR67}, a 41kDa Gi-like or 67kDa G-proteins that are associated with the insulin receptor; Gs, a stimulatory G-protein; IRS-1, insulin receptor substrate-1; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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corresponding to a part of the tyrosine kinase domain of the insulin receptor (amino acids 1135-1156), directly interacts with and stimulates GTPγS binding to the G_{IR67} (14).

A 40 to 41kDa Giα-like protein also has been shown to be regulated by insulin. Many of the biologic effects of insulin are inhibited by pertussis toxin (4,6-9). Conversely, insulin inhibits pertussis toxin-catalyzed ADP-ribosylation of Gi-like proteins in plasma membrane (3,7) and stimulates GTP binding to a similar protein (19). Here we report that insulin stimulates the association of the insulin receptor with a 41kDa G-protein, G_{IR41}.

MATERIALS AND METHODS

Treatment of adipocytes and preparation of membranes. Adipocytes were isolated from 30 or 50 rats (male, Sprague-Dawley, 100-150g) as described (20). Cells were washed in Krebs-Ringer's phosphate buffer (pH 7.4) containing 3% bovine serum albumin and 1.5 mM pyruvate (KRP-1). Following the resting period of 1 h at 37°C, insulin (diluted in KRP-1) or KRP-1 alone (control) were added at 37°C as specified in Figure legends. Adipocytes were washed and homogenized at 14°C in buffer A (10mM Tris-HCl, pH 7.4, 0.25M sucrose) by the method of Jarett (21). The homogenate was pelleted at 20,000 x g for 15 min at 4°C. The pellet was homogenized again in a 2 ml of buffer A, and centrifuged at 1,000 x g for 10 min at 4°C. The supernatant (crude membranes) containing plasma membrane and mitochondria were used immediately for immunoprecipitation experiments or stored at -70°C until used for other experiments.

ADP-ribosylation of crude membranes. Crude membranes (50μg each) prepared from insulin treated and control adipocytes were ADP-ribosylated in 100μl assay volume containing 5μg of pertussis toxin [pre-activated with 0.5mM dithiothreiotol (DTT)], 50μM NAD and 10 μCi [^{32}P]NAD for 30min at 30°C as described (7). The reaction was stopped by adding Laemmli's sample buffer, boiled for 5min, separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a PVDF membrane as described (3). The blotted membrane was rinsed in Tris buffered saline, dried and autoradiographed.

Co-immunoprecipitation of the insulin receptor and a 41kDa G-protein. The immunoprecipitation procedure was performed at 4°C. Equal amounts of crude membranes (200 to 400μg) prepared from insulin-treated or control adipocytes were centrifuged at 20,000 x g for 15min. Pelleted membranes were solubilized in 500μl of IP wash buffer (50mM HEPES, pH 7.4, 0.15M NaCl, 1mM vanadate, 1% Triton x-100,10 % glycerol and 1mM phenylmethyl sulphonyl fluoride) for 1h and centrifuged at 20,000 x g for 15min. Solubilized membranes were incubated with 5μg of non-immune mouse (control) IgG1 pre-absorbed to 20μl of Protein G-agarose [50%(v/v) suspension in IP wash buffer] for 30min on a rotator and centrifuged at 14,000rpm for 1min. The supernatants were then incubated with 5μg of either control IgG1 or insulin receptor antibody, AB-3 (Oncogene, Uniondale, NY), for 2h followed by a 30min incubation with 20μl of Protein G-agarose slurry. The agarose beads were washed four times with IP wash buffer and once with 50mM Tris buffer, pH 7.4 containing 0.1% Triton X-100. To quantitate the amount of the 41kDa G-protein in immunoprecipitates, beads were ADP-ribosylated in a 50μl assay volumes containing 5μg of pertussis toxin, (pre-activated with 20mM DTT), 50μM NAD and 20 μCi [^{32}P]NAD for 1h at 30°C. The reaction was stopped by adding Laemmli's sample buffer (22), boiled for 10min, separated on a 10% SDS-PAGE, electrotransferred and autoradiographed as described in the above. After obtaining an autoradiogram, each PVDF membrane

was probed with either an insulin receptor antibody [CT-1 from Ken Siddle, (23)] or a Giα antibody [UBI, Lake Placid, NY, (3)].

Other methods. Protein concentration was measured by a Biorad DC assay using bovine serum albumin as a standard. Autoradiograms and immunoblots were quantitated by densitometry using a LYNX system (Applied Imaging, Santa Clara, CA). Results shown here have been reproduced at least three times.

RESULTS

The pertussis toxin-catalyzed ADP-ribosylation of a 41kDa G-protein in crude membranes prepared from isolated rat adipocytes that had been treated with increasing concentrations of insulin was characterized (Figure 1). Insulin inhibited ADP-ribosylation of a 41kDa G-protein in a concentration-dependent manner within the physiologically-relevant range of insulin concentrations.

Previously we showed evidence that the G_{IR41} and G_{IR67} are associated with the insulin receptor (3,14). Therefore, we tested whether insulin could affect the association of these two G-proteins with the insulin receptor. The amount of ADP-ribosylation of the G_{IR41} co-immunoprecipitating with the insulin receptor from rat adipocytes was increased in an insulin concentration-dependent manner. An insulin receptor antibody (AB-3), but not a control IgG1 [(NIM), results not shown], specifically immunoprecipitated the insulin receptor from solubilized membranes and insulin treatment of adipocytes did not change the amount of the receptor precipitated [Figure 2B (α-IR blot)]. In contrast, the amount of the G_{IR41} detected by pertussis toxin-catalyzed ADP-ribosylation of the immunoprecipitates was increased by increasing concentrations of insulin (Figure 2B, PTX). This result was not due to a non-specific co-precipitation of 41kDa G-proteins as control IgG1 immunoprecipitates did not show

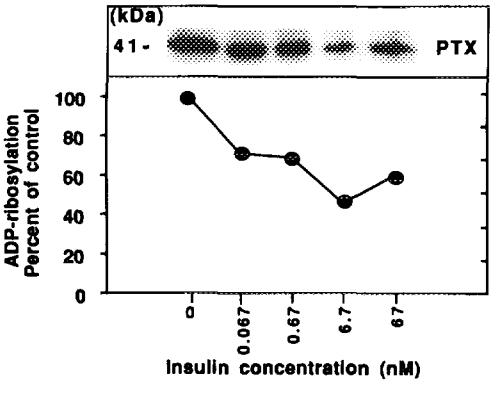

Fig. 1. **Insulin inhibits pertussis toxin-catalyzed ADP-ribosylation of a 41kDa G-protein in adipocyte membranes.** Crude membranes were prepared from isolated rat adipocytes that were treated with vehicle alone (0) or with increasing concentrations of insulin for 10 min at 37°C. Crude membranes (50μg each) were ADP-ribosylated in the presence of pertussis toxin , separated by SDS-PAGE and autoradiographed (upper panel). The density of the ADP-ribosylated 41kDa protein in the upper panel was quantitated by densitometry (lower panel).

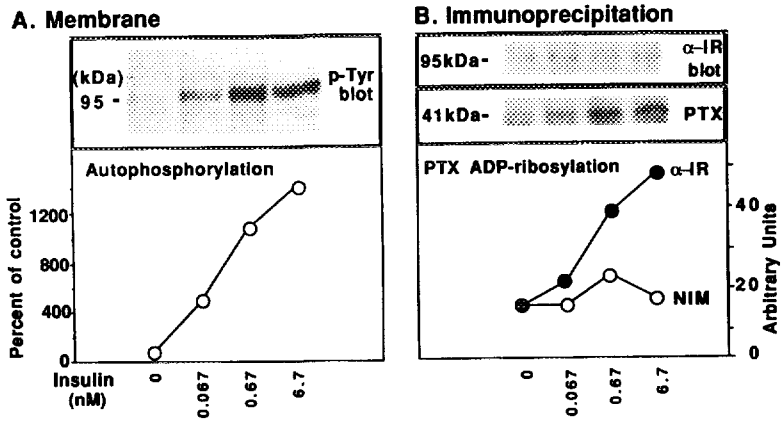

Fig. 2. Insulin stimulates co-immunoprecipitation of the G_{IR41} with the insulin receptor in a concentration-dependent manner. Crude membranes were prepared from adipocytes that were treated with increasing concentrations of insulin and divided into three aliquots. Panel A: One aliquot (50μg each) of each crude membrane preparation was directly separated on a SDS-PAGE, blotted and probed with phosphotyrosine antibody. The graph shown in the lower box of the panel A is a quantitation of the result shown in the upper box. Panel B: The remaining two aliquots (200μg each) of each crude membrane preparation were solubilized and immunoprecipitated with either AB-3 (α-IR) or control IgG1 (NIM). Immunoprecipitates were then ADP-ribosylated in the presence of pertussis toxin, separated by SDS-PAGE and autoradiographed (PTX). After obtaining an autoradiogram (41kDa, PTX), the PVDF membranes were immunoprobed with CT-1 antibody (95kDa, α-IR blot). A blot showing the β-subunit of the insulin receptor (top) and an autoradiogram of ADP-ribosylated 41kDa G-protein (middle) are shown. The graph in the lower box is the quantitation of the results of the ADP-ribosylation experiment from both AB-3 antibody and NIM .

a significant amount of ADP-ribosylated 41kDa G-protein (Figure 2B, PTX ADP-ribosylation, NIM). The insulin-stimulated increase in the G_{IR41} associated with the insulin receptor closely paralleled the insulin-dose dependency of the stimulation of autophosphorylation of the insulin receptor (Figure 2A). In parallel experiments, we tested whether the G_{IR67} co-immunoprecipitated with the insulin receptor using both western blot with a common Gα antibody and GTP crosslinking as methods of detection (3). Under these experimental conditions, the G_{IR67} did not co-precipitate with the insulin receptor (data not shown). This may be because the insulin receptor has a low affinity for the G_{IR67}. Alternatively, the G_{IR67} (14) and AB-3 antibody (24) may share similar recognition sites in the cytoplasmic domain of the insulin receptor so that the antibody can only immunoprecipitate the insulin receptor that are not bound to the G_{IR67}.

Next, the effect of time of incubation with insulin on the association of the G_{IR41} with the insulin receptor was examined. As expected, insulin stimulated autophosphorylation of the insulin receptor in a time-dependent manner reaching maximum by 2 min (Figure 3A). Insulin also stimulated the amount of ADP-ribosylated G_{IR41} that co-immunoprecipitated with the insulin receptor in a time-dependent manner. The effect of insulin was a continuing increase for the entire 10min

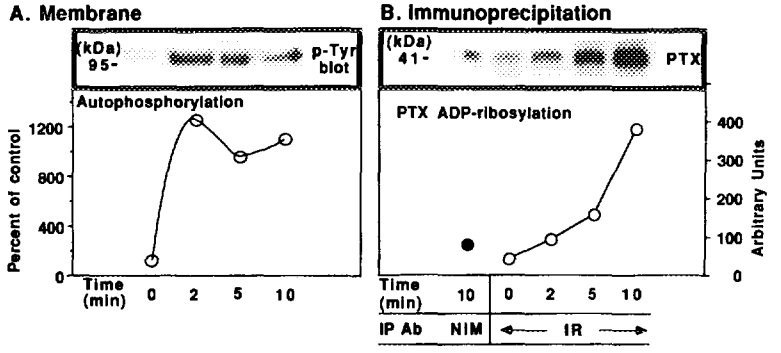

Fig.3. Insulin stimulates co-immunoprecipitation of the G_{IR41} with the insulin receptor in a time-dependent manner. Crude membranes were prepared from adipocytes that were treated with 1nM insulin for 0 to 10min and divided into aliquots as in Figure 2. Panel A: Crude membranes (50μg each) were directly immunoblotted with phosphotyrosine antibody (upper box) and quantitated values from these are plotted in the lower box. Panel B: Aliquots of each crude membrane preparations (400μg) were immunoprecipitated with AB-3 antibody (IP Ab: IR) and an additional aliquot (400μg) of crude membranes obtained from 10min insulin-treated adipocytes were treated with control IgG1 (NIM). Immunoprecipitates were ADP-ribosylated, separated by a SDS-PAGE, blotted, autoradiographed (upper box) and quantitated (lower box) as in Figure 2. The closed circle represents the value obtained with NIM, whereas the open circles are values obtained with AB-3 antibody at each insulin incubation time.

incubation period. The time course for insulin-mediated association of the G_{IR41} with the insulin receptor, although rapid, is clearly slower than the time course for insulin-stimulated autophosphorylation of the insulin receptor. This may indicate that tyrosine phosphorylation and/or conformational change of the insulin receptor has to occur before the 41kDa G-protein can bind to the receptor.

DISCUSSION

Previous observations support the concept that the insulin receptor is coupled to a Gi-like protein. Muller-Wieland et al., (4) have shown functional evidence that pertussis toxin-sensitive G-protein (presumably a Gi-protein) inhibits insulin-stimulated autophosphorylation of the insulin receptor and pp185 phosphorylation (presumably IRS-1) without changing insulin binding. Insulin-stimulated glucose transport (6,8), antilipolytic activity (25), thymidine uptake (8) and inositol phosphoglycan turnover (9) have also been known to be inhibited by pertussis toxin. Moreover, it has been shown that insulin treatment of either adipocytes or plasma membranes 1) inhibits pertussis toxin-catalyzed ADP-ribosylation of a 41kDa G-protein in membranes (3,7) without changing the total mass of Gi-proteins in the plasma membrane (3,26) and 2) stimulates GTP-binding to a 40kDa Gi-like protein (19). There is no evidence in intact cells that insulin induces post-translational modification of this Gi-protein, such as tyrosine phosphorylation or ADP-ribosylation (4,7,27), although the purified insulin receptor can phosphorylate purified G-protein (28). Rothenberg and Kahn suggested

that direct non-covalent interactions of the insulin receptor and G-proteins or other membrane components may occur (7). Consistent with their suggestion, Record et al. (26), recently reported that insulin alters immunodetectability of a Giα protein in rat adipocytes. They interpreted this to indicate an insulin-induced change in the conformation of the carboxy terminus of a Gi-protein by coupling to the insulin-stimulated insulin receptor. Moreover, we recently showed evidence that the G_{IR41} and G_{IR67} are associated with the insulin receptor (3,14). Therefore, we examined the possibility that inhibitory effect of insulin on pertussis toxin-catalyzed ADP ribosylation of a 41kDa G-protein may be due to a physical binding of the G-protein to the insulin receptor.

To our surprise, there is a paradoxical effect of insulin on pertussis toxin-catalyzed ADP-ribosylation of a 41kDa Gi-like protein. Consistent with a previous report (3) , insulin treatment of adipocytes inhibited pertussis toxin-catalyzed ADP-ribosylation of a 41kDa G-protein in subsequently prepared membranes. The insulin concentration dependency of this effect is within the physiologically relevant range (Figure 1). To the contrary, insulin treatment of adipocytes simultaneously increased the association of a 41kDa G-protein co-precipitating with the insulin receptor (Figure 2 and 3). The paradoxical effect of insulin is not likely due to Triton X-100 used in immunoprecipitation experiments since it was shown that insulin-induced inhibition of ADP-ribosylation of a 41kDa G-protein was not altered by the presence of Triton X-100 detergent (7; data not shown). The underlying molecular mechanisms for this paradoxical effect of insulin are not clear.

Interestingly, a similar paradoxical phenomenon has been reported for the EGF receptor. EGF inhibits pertussis toxin-catalyzed ADP-ribosylation of a Gi-protein in pancreatic acinar cell membranes (29) and stimulates association of the EGF receptor with a 41kDa G-protein as detected by pertussis toxin-catalyzed ADP-ribosylation in hepatocytes (30). One should be cautioned, however, that those studies as well as the current study lack independent evidence (i.e., western blotting with antibodies to Giα or Gβ-subunit) that the insulin-induced increase in ADP-ribosylation of receptor-bound Gi-like proteins (Figure 2 and 3) is due to an increase in the mass of the 41kDa α-subunit and/or the βγ-subunit of the G-protein bound to the EGF or insulin receptors. In our study, immunoblot with antibodies to a common Gα or Giα antibodies failed to recognize a Giα-like protein in the insulin receptor co-precipitates (data not shown). Possible reasons for this include: a) lack of sufficient sensitivity to detect the amount of G_{IR41} co-immunoprecipitating with the receptor or b) the G_{IR41} is unique and is not detected by the G-protein antibodies used.

We recently identified a G-protein binding region in the tyrosine kinase domain of the insulin receptor (1135-1156) containing three tyrosine residues that become autophosphorylated (14). In that study the effect of the synthetic non-phosphorylated peptide (1135-1156) on the two insulin receptor associated G-proteins, G_{IR41} and G_{IR67} was tested (3). The non-phosphorylated peptide stimulated GTPγS binding to only the 67kDa G-protein but not to the 41kDa G-protein (14). This finding combined

with our present results suggests that the non-activated insulin receptor binds to the G_{IR67} but poorly with the G_{IR41}. However, activation of the insulin receptor may stimulate association of the G_{IR41} to the receptor. It will be extremely interesting to test whether the G_{IR41} or G_{IR67} forms a complex with the IRS-1 or IRS-1 binding proteins.

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