The G972R variant of the insulin receptor substrate-1 gene impairs insulin signaling and cell differentiation in 3T3L1 adipocytes; treatment with a PPARγ agonist restores normal cell signaling and differentiation

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Abstract

The insulin receptor substrate-1 (IRS-1) plays a central role in insulin sensitivity, and association studies have shown that the IRS-1 G972R variant is a risk factor for insulin resistance. However, how this mutation may lead to impaired insulin sensitivity is still to be determined. Our study aimed to evaluate, after transfection of the IRS-1 G972R variant in 3T3L1 adipocytes, the effect of this mutation on insulin signaling and on cell differentiation. The 3T3L1 cells were transfected with pcDNA3 expression vector containing either the human wild-type IRS-1 or the G972R variant. After induction of differentiation, the 3T3L1 transfected with wild-type IRS-1 differentiated in 6–8 days, while the cells transfected with G972R variant did not differentiate. To determine whether the defect in IRS-1 was responsible for this, we analyzed the expression of several genes involved in the insulin signaling pathway. Results showed that PPARγ expression was significantly reduced in cells transfected with the mutated IRS-1, together with a significant decrease in binding of phosphatidylinositol-3 kinase (PI 3-kinase) to IRS-1 G972R and in PI 3-kinase activity. In addition, we observed that the interaction between the insulin receptor (IR) and the IRS-1 G972R protein was increased and that the autophosphorylation of the IR was significantly inhibited in 3T3L1-G972R cells compared with 3T3L1-WT. Treatment of the 3T3L1-G972R cells with pioglitazone (PIO), a PPARγ agonist, restored differentiation with higher level of PPARγ expression and restoration of PI 3-kinase binding to IRS-1 G972R and PI 3-kinase activity. IR autophosphorylation was also increased. Withdrawal of PIO in fully differentiated 3T3L1-G972R cells determined the reappearance of the insulin signaling defect. Finally, we observed higher levels of IRS-2 expression, suggesting that IRS-2 may play a more important role in adipocyte insulin signaling. In conclusion, IRS-1 G972R variant impairs insulin signaling, and treatment with PPARγ agonist restores the normal phenotype of 3T3L1 cells.

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Introduction

In the pathogenesis of common disorders, such as type 2 diabetes mellitus, hypertension and atherosclerosis, a central role is played by insulin resistance. In the search for susceptibility loci predisposing to insulin resistance, previous studies have identified a glycine to arginine substitution at codon 972 (G972R) in the insulin receptor substrate-1 (IRS-1) gene as being associated with impaired insulin action, particularly in obese patients (Clausen *et al.* 1995, Baroni *et al.* 2001). The IRS-1 gene, which plays a pivotal role in the signaling cascade activated by insulin, is therefore a strong candidate for the genetic susceptibility to insulin resistance.

After the binding of insulin to its receptor, the intrinsic tyrosine kinase activity of the receptor β-subunit is activated, thus catalyzing the phosphorylation of specific tyrosine residues on the IRS-1 protein. Phosphorylated IRS-1 binds to several cellular signal proteins, thus functioning as a multisite 'docking' protein linking the receptor kinase to the variety of cell functions regulated by insulin (Meyers & White 1993). In particular, phosphorylated IRS-1 binds with high affinity to proteins with Src homology-2 domains, including phosphatidylinositol-3 kinase (PI 3-kinase), a lipid kinase composed by a regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) (Sakaue *et al.* 1998). Activated PI 3-kinase generates phospholipids that act by multiple mechanisms and that cooperate to regulate

Akt activity. The PI 3-K/Akt cascade has been implicated in various insulin-dependent cellular processes, including cell survival, glucose transport and adipocyte differentiation (Magun *et al.* 1996, Franke *et al.* 1997, Ueki *et al.* 1998). A consequence of Akt binding to phospholipids is the translocation of Akt from the cytoplasm to the inner surface of the plasma membrane and its change of conformation, so that Thr[308] and Ser[473] residues become accessible for phosphorylation by the phosphoinositide-dependent protein kinases PDK1 and PDK2. Activation of Akt also results from the phosphorylation at the two sites (Datta *et al.* 1999, Alessi 2001). However, the mechanisms by which the PI 3-K/Akt cascade is relayed to the nucleus to activate adipocyte differentiation are not well understood. In orchestrating adipocyte differentiation, the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ), the CCAAT/enhancer-binding proteins (C/EBPs), and adipocyte determination and differentiation-dependent factor 1 (ADD-1) are key transcription factors (Kim & Spiegelman 1996). PPARγ is induced early during the course of adipocyte differentiation, binds to specific DNA-response elements as a heterodimer with the RXR proteins, and plays a key role in adipocyte differentiation (Auwerx 1999). Another pathway activated by the tyrosine phosphorylation of IRSs by the insulin receptor (IR) is the mitogen-activated protein kinases (MAP kinases) pathway. MAP kinases belong to a group of protein-serine/threonine kinases that are activated in response to various stimuli (growth factors, neurotransmitters, differentiating agents and heat shock) and are involved in proliferation and differentiation processes, in particular by regulating the transcriptional activity of the nucleus (Porras *et al.* 1998).

To define the role of the G972R variant of IRS-1 gene in insulin signaling, we have transfected this variant in 3T3L1 adipocytes, and examined the proteins involved in IRS-1-mediated signaling, including those implicated in adipocyte differentiation. Our results demonstrate impairment in the differentiation process in 3T3L1-G972R, associated with several alterations of the insulin-signaling pathway, including a significant reduction of PPARγ expression. We also investigated the effects of a PPARγ agonist, pioglitazone (PIO), on the molecular alterations in IRS-1-mediated signaling associated with the G972R variant. PPARγ agonists, also known as thiazolidinediones (TZDs), are a class of antidiabetic agents that act by improving insulin sensitivity (Nolan *et al.* 1994, Zierath *et al.* 1998). TZDs exert their antidiabetic actions by various mechanisms: enhancing insulin signaling, increasing glucose transporters and uptake, lowering plasma FFA, reducing hepatic glucose production, decreasing tumor necrosis factor-α levels in muscle and increasing plasma adiponectin levels (Maeda *et al.* 2002). PPARγ agonists are direct ligands for PPARγ (Spiegelman 1998), and promote fat cell differentiation by the activation of several adipocyte-specific genes.

Materials and Methods

Cell culture condition and treatment

Murine 3T3L1 preadipocytes were maintained in DMEM/high-glucose medium supplemented with piruvic acid (110 mg/l), sodium hydrogen carbonate (1·5 g/l at 5% CO_2) and 10% calf serum. Differentiation of stable transfected 3T3L1 was induced by exposing confluent cells to insulin (1 µM), isobutylmethylxanthine (0·5 mM), and dexamethasone (0·25 µM) (all from Sigma) for 2 days, and then to insulin (1 µM) alone for another 2 days. The medium was changed every 2 days until day 8. The TZD compound PIO (kindly supplied by Takeda Chemical Industries, Osaka, Japan) was added at 1 µM from the induction step of differentiation, and cells were cultured with PIO until full differentiation. For long-term PIO treatment, fully differentiated adipocytes at day 8 of differentiation were incubated for an extra 48 h in the presence or absence of 10 µM PIO followed by stimulation with 100 nM insulin. For time-course study, 3T3L1 cells were placed in DMEM supplemented with 2% calf serum for (16–20 h) and then stimulated for the indicated times (0–40 min) at 37 °C in the presence of 100 nM insulin.

Oil Red O staining

Cells were washed three times with PBS, fixed by 10% formalin in PBS for 1 h at room temperature, washed once again with PBS, and then stained with 60% filtered Oil Red O stock solution (0·5 g Oil Red O (Sigma) in 100 ml isopropanol) for 20 min, washed four times with water and analyzed under a microscope.

Construction and expression of human IRS-1 cDNAs in 3T3L1 cells

pcDNA3 expression vectors (Invitrogen) containing human wild-type (WT) IRS-1 or the G972R variant were constructed as previously described (Imai *et al.* 1997). 3T3L1 cells were stably transfected with 1 µg pcDNA3 alone, WT pcDNA3 IRS-1 or pcDNA3 G972R IRS-1, and suspended with 20 µl lipofectamine in 1 ml serum-free DMEM (Invitrogen). The cells were incubated with a selective medium containing 0·4 mg/ml geneticin G-418 sulfate (Invitrogen). From all the G-418-resistant stable cell clones obtained from the transfection (seven clones with the WT IRS-1 and five clones with the G972R variant), we selected clones expressing similar amounts of IRS-1 protein by analyzing lysates by Western blotting. We selected also clones with the G972R variant expressing high and low amounts of IRS-1 protein. We performed all the experiments reported in the paper with three clones expressing WT IRS-1 and with two clones

expressing the G972R variant. Additionally, we performed experiments in two clones expressing the empty vector.

IR and IRS-1 tyrosine phosphorylation and association with the p85 subunit of PI 3-kinase

The transfected 3T3L1 were placed in DMEM containing 2% calf serum (16–20 h) and then incubated for 5 min at 37 °C in the presence or absence of 100 nM insulin. The cells were then lysed for 45 min at 4 °C with lysis buffer (137 mM NaCl, 20 mM Tris–HCl (pH 7·6), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EDTA, 10% glycerol, 10 mM $Na_4P_2O_4$, 100 µM sodium orthovanadate, 10 mM NaF, 10 µg/ml leupeptin, 2 mM phenylmethyl-sulfonylfluoride and 1·5% Nonidet P-40). Insoluble materials were removed by centrifugation, and the super-natants were incubated with polyclonal anti-IRS-1 or anti-IR antibody (Upstate Biotechnology, Lake Placid, NY, USA), followed by incubation with protein A-Sepharose for 2 h at 4 °C. The immunoprecipitated proteins were resuspended in Laemmli buffer, resolved by SDS–PAGE and transferred onto nitrocellulose membrane (Hybond ECL, Amersham). The membranes were incu-bated with polyclonal antiphosphotyrosine antibody (Transduction Laboratories, Lexington, KY, USA) or with anti-PI 3-kinase p85 (Upstate Biotechnology), followed by incubation with HRP-conjugated secondary antibodies. Immunoreactive bands were detected with the ECL kit (Amersham) and Biomax Light Kodak. Quantification was determined by densitometric scan-ning. All blots were normalized for the amount of immunoprecipitated proteins.

IRS-1-associated PI 3-kinase activity

Transfected 3T3L1 were placed in DMEM containing 2% calf serum (16–20 h) and then incubated for 5 min at 37 °C in the presence or absence of 100 nM insulin. The cells were then lysed for 20 min with 1 ml ice-cold lysis buffer (137 mM NaCl, 20 mM Tris–HCl (pH 7·4), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0·1 mM sodium orthovanadate, 1% Nonidet P-40 and 1 mM phenylmethylsulfonylfluoride). An amount of 50 µg whole-cell lysate was incubated with anti–IRS-1 antibody, followed by incubation with protein A-Sepharose for 2 h at 4 °C, and washed with TNE buffer (10 mM Tris–HCl (pH 7·4), 150 mM NaCl, 5 mM EDTA and 0·1 mM sodium orthovanadate). The PI 3-kinase activity was studied in immunoprecipitates with a PI 3-kinase ELISA kit (Echelon Biosciences, Salt Lake City, UT, USA).

Immunoblotting

Antibodies against the serine 473/474, threonine 308/309 phosphorylated regions, and the nonphosphorylated regions of Akt1 and Akt2, antiphospho-PDK1 (Ser[241])

polyclonal antibody, and antiphospho-p42/p44 MAP kinases monoclonal antibody were all from Cell Signaling Technology (Beverly, MA, USA). Polyclonal anti-IRS-2, anti–phospho-FKHRL1 (Thr[32]), and monoclonal anti-PPARγ antibodies that react with both PPARγ1 and PPARγ2 forms, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

An amount of 30–40 µg total cell lysates, determined by the Bradford assay, were separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were incubated with the specific primary antibody followed by incubation with HRP-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence, and densities of blots were quantified by densitometric scanning. Percent differences in protein binding and expression were calculated by computing the percent reduction/increase relative to that in WT cells (binding/expression in WT: 100 = binding/expression in G972R:x). Where necessary, immunoblots were stripped of anti-bodies, according to the manufacturer's instructions, and reprobed with different antibody. To ensure removal of antibodies, membranes were then incubated with ECL (Amersham) detection reagents and exposed to film.

Statistical analysis

Differences in the expression of mRNA and protein levels between the cell lines were analyzed by unpaired or paired Student's *t*-test where appropriate. A *P* value of less than 0·05 was considered statistically significant.

Results

Adipocyte differentiation in 3T3L1-G972R cells is impaired

3T3L1 cells were stably transfected with pcDNA3 expression vectors containing human WT IRS-1 or the G972R variant. In all transfected cell clones used in this study, expression of WT IRS-1 and of G972R variant was about 1·9-fold–higher than that of the endogenous IRS-1, as detected by immunoblotting. Thus, similar amounts of recombinant IRS-1 were observed in 3T3L1-WT and 3T3L1-G972R cells (data not shown).

Differentiation in transfected 3T3L1 was induced as described in Materials and Methods. After induction, preadipocytes carrying WT IRS-1 rapidly accumulated fat, and a fully differentiated phenotype with 80–90% of the cells containing multilocular fat droplets could be observed 6 days after induction (Fig. 1A). In contrast, only 20–30% of the 3T3L1 cells carrying the G972R variant were able to accumulate fat (Fig. 1B). Moreover, 3T3L1 cells transfected with pcDNA3 alone (empty vector) differentiated normally in 6–8 days, confirming that no significant cytotoxicity was caused by the control transfection (data not shown).
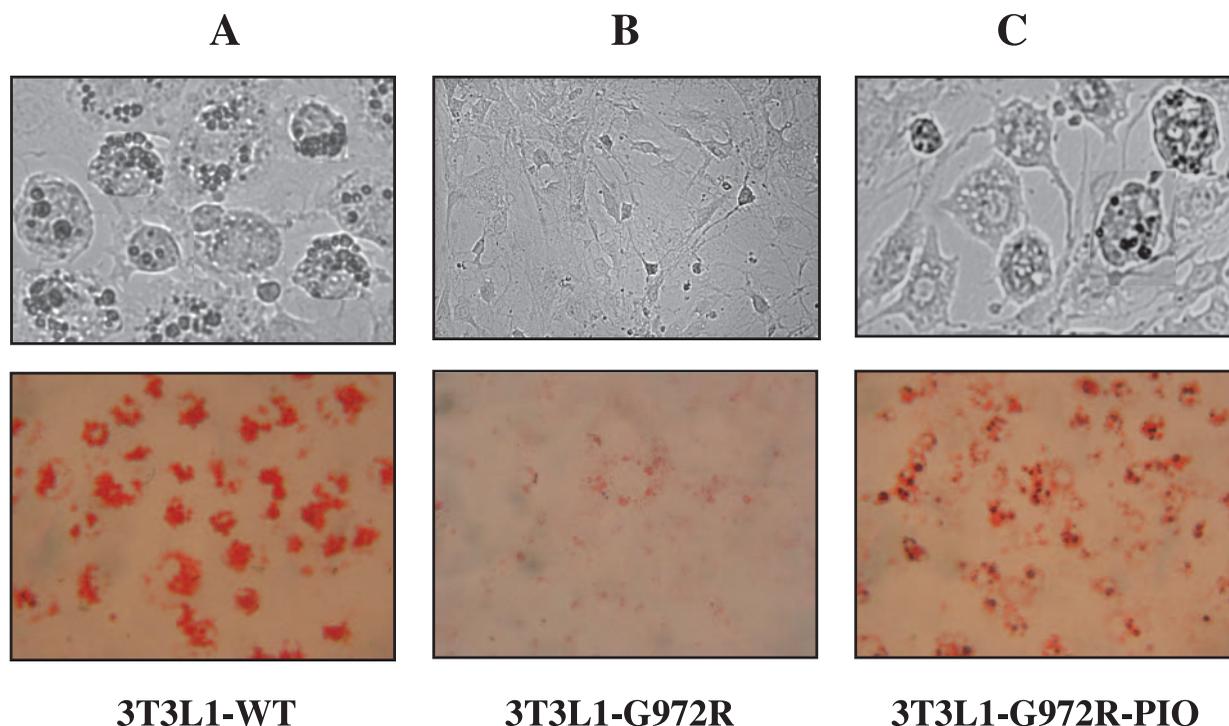
A   **B**   **C**

3T3L1-WT   **3T3L1-G972R**   **3T3L1-G972R-PIO**

Figure 1 Effect of the G972R variant of IRS-1 gene on differentiation of 3T3L1 cells into adipocytes (upper and lower panel). 3T3L1 transfected with wild-type (A) and G972R variant (B) were induced to differentiate by exposing confluent cells to insulin, isobutylmethylxanthine and dexamethasone, as described in Materials and Methods. (C) Pioglitazone (PIO) was added at 1 μM from the induction step of differentiation, and 3T3L1-G972R cells were cultured in the presence of PIO until full differentiation. The degree of adipogenic differentiation was evaluated by Oil Red-O staining at day 8 of differentiation (20) (lower panel).

To determine whether the G972R variant of the IRS-1 gene could cause the defective differentiation process in 3T3L1 cells, we assessed the expression of PPARγ during adipocyte differentiation by immunoblotting and RT–PCR. In 3T3L1-WT cells, both forms of PPARγ, γ1 and γ2, are low at day 0 and increase as expected during the differentiation process (Fig. 2A). Conversely, in 3T3L1-G972R cells, the expression of PPARγ gene was greatly reduced (Fig. 2B). Compared with 3T3L1-WT cells, PPARγ expression in 3T3L1-G972R cells was 4·1-fold lower at day 2 of differentiation ($P<0·03$), 3·8-fold lower at day 4 ($P<0·016$), 3·1-fold lower at day 6 ($P<0·011$) and 3·5-fold lower at day 8 ($P<0·01$).

Furthermore, the expression by RT–PCR of specific adipocytic differentiation markers, such as adiponectin (adipoQ) and adipsin, was reduced in the 3T3L1-G972R cells compared with the 3T3L1-WT cells (data not shown).

IRS-1 tyrosine phosphorylation and binding of p85 subunit of PI 3-kinase

To clarify the possible mechanisms by which the G972R variant could affect insulin signaling, we studied tyrosine phosphorylation of IRS-1 and its binding to the p85 subunit of PI 3-kinase. Equal amounts of total protein from transfected 3T3L1 cells, either stimulated or not with insulin, were immunoprecipitated with anti-IRS-1 antibody, subjected to SDS–PAGE, and immunoblotted with antiphosphotyrosine or anti-PI 3-kinase p85 antibodies (Fig. 3A and B). Basal phosphorylation of IRS-1 in unstimulated cells showed no significant difference between 3T3L1-WT and 3T3L1-G972R cells. Quantitation of the protein bands, after correction for the amount of IRS-1 protein level immunoprecipitated, showed that, after insulin stimulation, IRS-1 phosphorylation was increased by 1·5-fold ($P<0·02$) in 3T3L1-WT and 1·5-fold ($P<0·02$) in 3T3L1-G972R, compared with the basal in each cell line. In 3T3L1-G972R cells, insulin-stimulated phosphorylation of IRS-1 was 17% lower than with 3T3L1-WT ($P<0·03$) (Fig. 3A).

Basal binding of p85 subunit to IRS-1 in unstimulated cells showed no significant differences between 3T3L1-G972R and 3T3L1-WT cells. Quantitation of the protein bands, after correction for the amount of immunoprecipitated IRS-1, confirmed that, after insulin stimulation, binding of p85 protein to IRS-1 was increased by 1·5-fold ($P<0·03$) in 3T3L1-WT and by 1·3-fold ($P<0·05$) in 3T3L1-G972R; thus, in 3T3L1-G972R, binding of p85
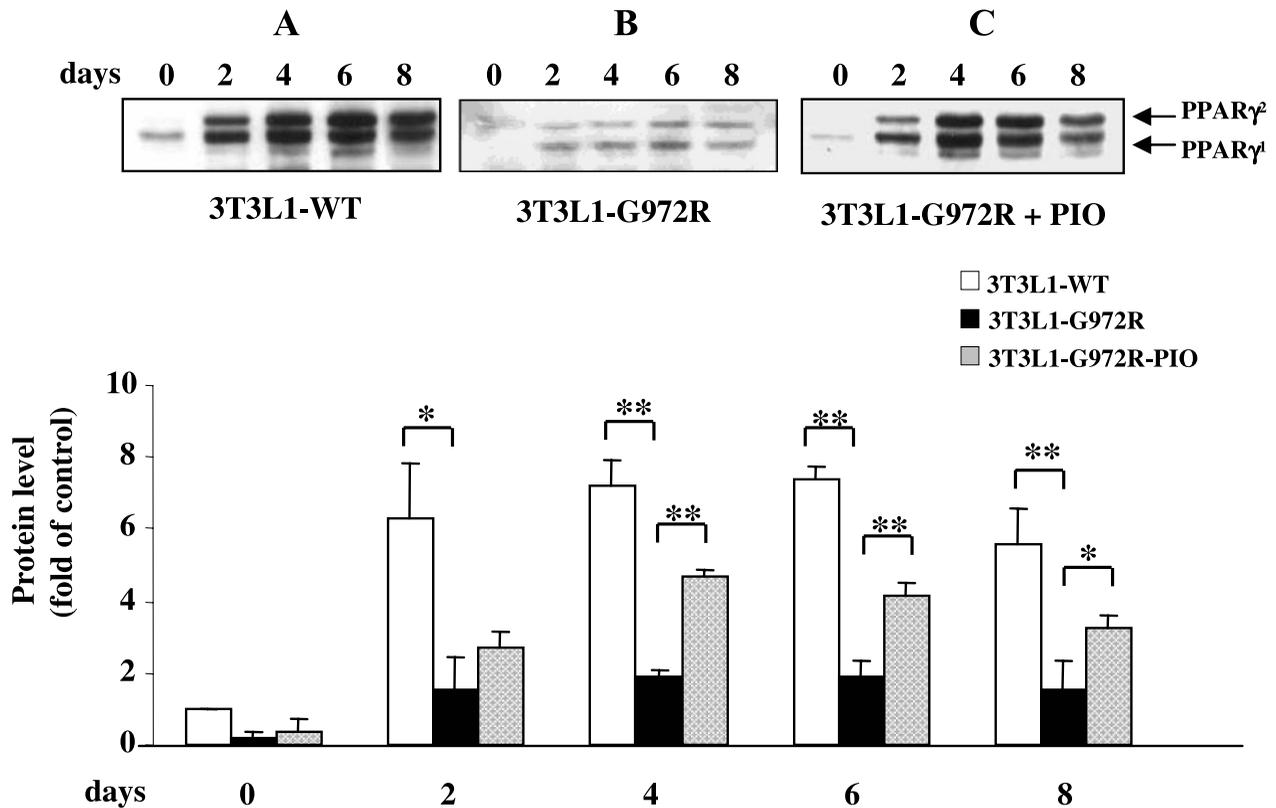
Figure 2 PPARγ expression during adipocyte differentiation. 3T3L1 with wild-type (A) and G972R variant (B) of the IRS-1 gene were differentiated, as described in Materials and Methods. Cell lysates were immunoblotted with anti-PPARγ antibody. (C) PIO was added at 1 µM from the induction step of differentiation, and 3T3L1-G972R cells were cultured in the presence of PIO until full differentiation. The protein levels of PPARγ were normalized by the value in control cells at day 0 of differentiation. Data represent three independent experiments. Only significant comparison between 3T3L1-G972R and 3T3L1-WT cells and between 3T3L1-G972R and 3T3L1-G972R-PIO cells are shown. *$P<0.05$; **$P<0.02$.

to IRS–1 was 25% lower ($P<0.02$) in response to insulin than in cells expressing the WT protein (Fig. 3B). The decrease was not due to a decrease in IRS–1 protein, as Western blots of lysates from 3T3L1 cells, either stimulated or not with insulin, revealed equal amounts of IRS–1 protein between the 3T3L1–WT and 3T3L1–G972R cells (Fig. 3, top panel).

PI 3-kinase activity in 3T3L1 cells expressing WT IRS1 or G972R variant

To determine whether the reduced interaction between IRS–1 and p85 protein in cells expressing the G972R variant resulted in reduced PI 3-kinase activity, equal amounts of total protein of transfected 3T3L1, stimulated or not with insulin, were immunoprecipitated with anti–IRS–1 antibody and subjected to kinase reactions, as described in Materials and Methods. Quantitation of the colorimetric signal of the ELISA plate revealed that 3T3L1–WT and 3T3L1–G972R cells had similarly low

levels of basal PI 3-kinase activity. After insulin stimulation, the IRS–1–associated PI 3-kinase activity was increased by 8·3-fold ($P<0.002$) in 3T3L1–WT and 4·6-fold ($P<0.03$) in 3T3L1–G972R cells. Therefore, in 3T3L1–G972R cells, PI 3-kinase activity was 35% lower ($P<0.004$) in response to insulin than in 3T3L1–WT cells (Fig. 4).

Insulin receptor (IR) tyrosine phosphorylation and interaction with IRS-1

A very recent study showed that the presence of the G972R polymorphism markedly enhances the association of the IRS-1 gene with the IR, allowing IRS-1 to act as an inhibitor of IR autophosphorylation (McGettrick *et al.* 2004).

To further investigate how the presence of the G972R polymorphism altered IRS-1 function, we studied the interaction between IRS-1 and the IR. Equal amounts of
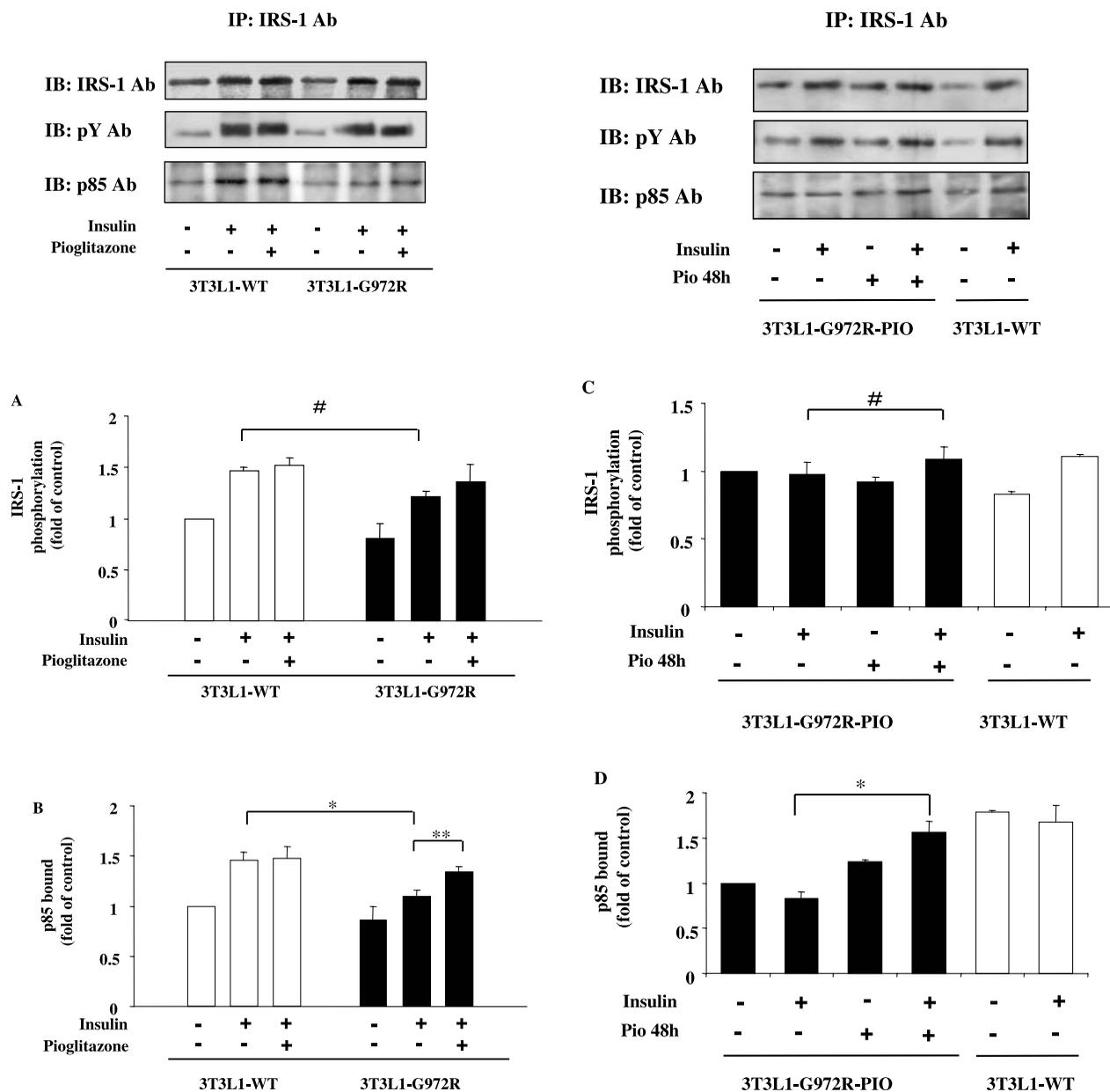
Figure 3 Tyrosine phosphorylation (A) and association of the p85 subunit of PI 3-kinase (B) of wild-type and G972R IRS-1 in 3T3L1 cell line. Top panel shows relative western blots. 3T3L1-WT or 3T3L1-G972R cells were incubated for 5 min in the presence or absence of 100 nM insulin, or for 48 h with 10 μM PIO followed by stimulation with insulin. Cell lysates were immunoprecipitated with anti-IRS1 antibody, and immunoblotted with antiphosphotyrosine antibody and with anti-PI 3-kinase p85. Results represent three independent experiments. Fold difference is by comparison with values in control cells. The relative levels of tyrosine phosphorylation and binding of the p85 subunit were quantified by densitometric scanning, and the values were divided by the values of IRS-1 protein immunoprecipitated. (A) #Significant decrease in 3T3 L1-G972R compared with 3T3L1-WT cells after insulin ($P<0.05$); (B) *significant decrease in 3T3L1-G972R compared with 3T3L1-WT cells after insulin ($P<0.05$); **significant increase in 3T3L1-G972R cells after PIO treatment ($P<0.05$). Tyrosine phosphorylation (C) and association of the p85 subunit of PI 3-kinase (D) in fully differentiated 3T3L1-G972R-PIO and in wild-type cells. Cells were incubated for an extra 48 h after day 8 of differentiation in the presence or absence of 10 μM PIO followed by stimulation with insulin. (C) #Significant decrease in 3T3L1-G972R-PIO with no 48-h PIO treatment compared with 3T3L1-G972R-PIO cells after 48-h PIO ($P<0.05$); (D) *significant decrease in 3T3L1-G972R-PIO without 48-h PIO treatment compared with 3T3L1-G972R-PIO cells after 48-h PIO ($P<0.005$).
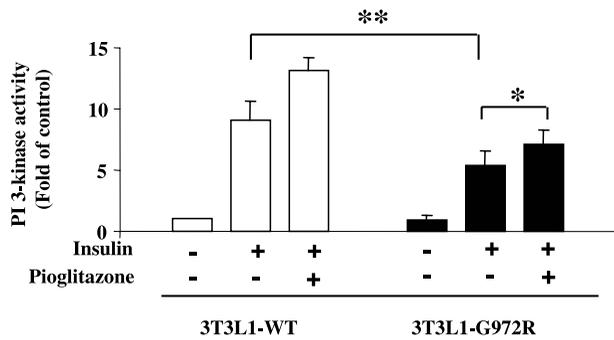
Figure 4 IRS-1 associated PI 3-kinase activity in 3T3L1 cells transfected with wild-type IRS-1 and G972R variant. Cells were incubated for 5 min in the presence or absence of 100 nM insulin or for 48 h with 10 µM PIO followed by stimulation with insulin. PI 3-kinase activity was quantified in immunoprecipitates with the ELISA plate reader. Data represent three independent experiments. Results are normalized for the values in control cells; **significant decrease in 3T3L1-G972R compared with 3T3L1-WT cells after insulin ($P<0.01$); *significant increase in 3T3L1-G972R cells after PIO treatment ($P<0.05$).

total protein from transfected 3T3L1 cells, either stimulated or not with insulin, were immunoprecipitated with antibody against the IR, subjected to SDS–PAGE, and immunoblotted with antiphosphotyrosine or anti-IRS-1 antibodies (Fig. 5). Quantitation of the protein bands, after correction for the amount of IR protein level immunoprecipitated, showed that basal binding of IRS-1 to the IR in unstimulated cells was 1·24-fold (24%) ($P<0.03$) higher in 3T3L1-G972R than in 3T3L1-WT cells. After insulin stimulation, binding of IRS-1 protein to the IR was increased by 1·1-fold ($P=$NS) in 3T3L1-WT and by 1·1-fold ($P=$NS) in 3T3L1-G972R cells; thus, in 3T3L1-G972R cells, binding of IRS-1 to the IR was 1·2-fold (24·5%) ($P<0.02$) higher in response to insulin than in cells expressing the WT protein (Fig. 5A).

Immunoblotting the same samples with antiphosphotyrosine antibody showed that insulin–stimulated tyrosine phosphorylation of the IR in 3T3L1-G972R was 52% of that in 3T3L1-WT ($P<0.003$) (Fig. 5B).

Serine and threonine Akt phosphorylation during differentiation of transfected 3T3L1

The activation of PI 3-kinase by insulin is an upstream step in stimulation of several serine/threonine kinases, including the proto–oncogene product Akt (also known as protein kinase B). Activation of Akt is considered an important step in adipocyte differentiation. We assessed the activation of Akt during adipocyte differentiation by immunoblotting with antibodies specific for both Thr[308] and Ser[473] phosphorylated forms of Akt. In 3T3L1-WT cells, high levels of serine and threonine Akt phosphorylation are present at day 0 of differentiation, but
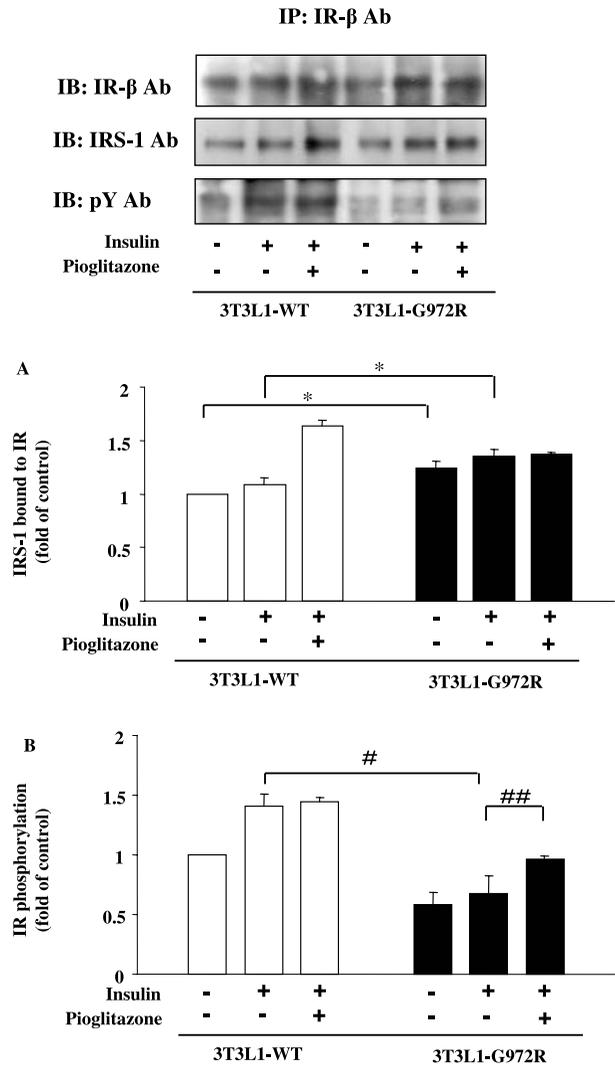

Figure 5 IR association with IRS-1 (A) and its tyrosine phosphorylation (B) in transfected 3T3L1 cell line. Top panel shows relative western blots. 3T3L1-WT or 3T3L1-G972R cells were incubated for 5 min in the presence or absence of 100 nM insulin, or for 48 h with 10 µM of PIO followed by stimulation with insulin. Cell lysates from 3T3L1-WT or 3T3L1-G972R cells were immunoprecipitated with anti-IR antibody, and immunoblotted with antiphosphotyrosine antibody and with anti-IRS-1. Results represent three independent experiments. Fold difference was calculated by comparison with values in control cells. The relative levels of tyrosine phosphorylation and binding of the IRS-1 protein were quantified by densitometric scanning, and the values were divided by the values of IR protein immunoprecipitated. (A) *Significant increase of IR-IRS-1 binding in 3T3L1-G972R compared with 3T3L1-WT cells before and after insulin ($P\leq0.03$). (B) #Significant decrease in 3T3L1-G972R compared with 3T3L1-WT cells after insulin ($P<0.003$); ##significant increase in 3T3L1-G972R cells after PIO treatment ($P<0.05$).

phosphorylation decreases during differentiation (Fig. 6A and B). However, in 3T3L1-G972R cells, the levels of Akt phosphorylation remained high during all times of
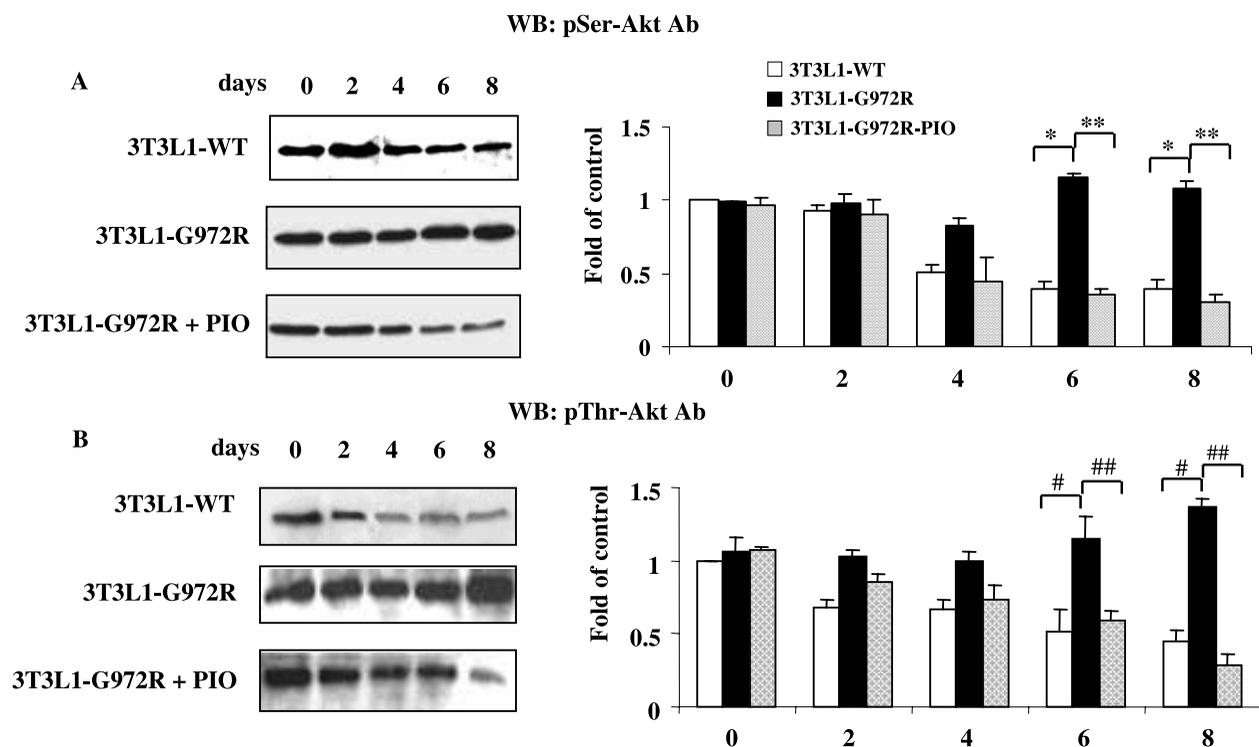
WB: pSer-Akt Ab

WB: pThr-Akt Ab

Figure 6 Akt serine (A) and threonine (B) phosphorylation during adipocyte differentiation. Cell lysates were immunoblotted with antiphospho-Akt(Ser^{473}) or antiphospho-Akt(Thr^{308}) antibody. PIO was added at 1 µM from the induction step of differentiation until full differentiation. Protein levels of phosphorylated Akt were normalized by the value in control cells at day 0 of differentiation. Data represent two independent experiments. Only significant comparisons between 3T3L1-G972R and 3T3L1-WT cells and between 3T3L1-G972R and 3T3L1-G972R-PIO cells are shown. (A) *Significant increase in 3T3L1-G972R compared with 3T3L1-WT cells ($P<0.05$); **significant decrease in 3T3L1-G972R-PIO compared with 3T3L1-G972R cells ($P<0.05$). (B) #Significant increase in 3T3L1-G972R compared with 3T3L1-WT cells ($P<0.05$); ##significant decrease in 3T3L1-G972R-PIO compared with 3T3L1-G972R cells ($P<0.05$).

adipocyte differentiation (Fig. 6A and B). Thus, compared with 3T3L1-WT cells, 3T3L1-G972R cells showed an increase by 1·6-fold at day 4 ($P=$NS), 2·8-fold at day 6 ($P<0.01$), and 2·7-fold at day 8 of differentiation ($P<0.03$) in the phosphorylation at Ser^{473}. The Akt phosphorylation at Thr^{308} showed a pattern comparable to Ser^{473}.

No change of Akt content could be observed between the different cell lines during differentiation (data not shown).

Insulin activates Akt through its phosphorylation by PDK1. By immunoblotting with an antibody specific for the Ser^{241} phosphorylated region of PDK1, we investigated whether the higher activation of Akt in 3T3L1-G972R cells could be associated with higher activation of PDK1. No significant difference in phosphorylation of PDK1 between the cell lines was observed (data not shown).

Despite the reduction in G972R IRS-1-associated PI 3-kinase activity, the G972R variant induces activation of Akt (in both Thr^{308} and Ser^{473} phosphorylated sites) more

strongly than WT IRS-1. Since Akt activation does not necessarily correlate with adipocyte differentiation but is both necessary and sufficient for cell survival and for the regulation of the apoptotic machinery (Datta *et al.* 1999), we assessed the activation of FKHRL1 during adipocyte differentiation by immunoblotting with antibody specific for the phosphorylated form of FKHRL1, which is a Forkhead family member of transcription factors involved in the apoptosis process and is phosphorylated by Akt. In the absence of phosphorylation, Forkhead family members, which are localized to the nucleus, bind to specific DNA elements that act as potent activators of gene involved in apoptosis. In 3T3L1-WT cell lines, high levels of FKHRL1 phosphorylation are present at day 0 of differentiation, but the levels of phosphorylation decrease during the differentiation process. However, in 3T3L1-G972R cells, the levels of FKHRL1 phosphorylation remain high during the process of adipocyte differentiation in homology with Akt. Thus, compared with 3T3L1-WT cells, 3T3L1-G972R cells showed 1·4-fold increase at day 4 of differentiation ($P<0.05$), 4·2-fold
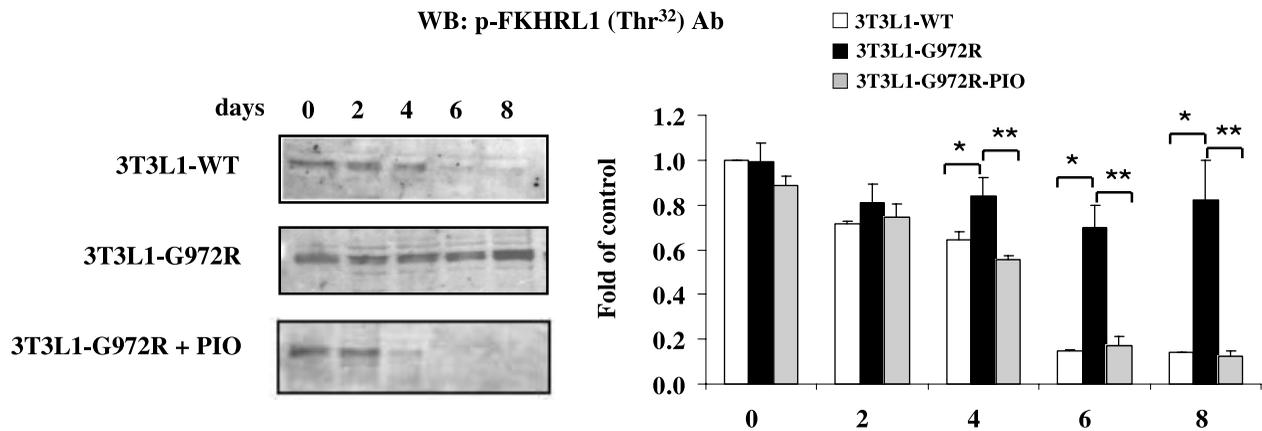
Figure 7 Induced activation of FKHRL1 during adipocyte differentiation in 3T3L1-WT and 3T3L1-G972R cells. PIO was added at 1 μM from the induction step of differentiation until full differentiation. Equal amounts of protein lysates at the indicated days of differentiation, as described in Materials and Methods, were separated by SDS–PAGE and immunoblotted with antibody specific for the phosphorylated form of FKHRL1. Protein levels of phosphorylated FKHRL1 were normalized by the value in control cells at day 0 of differentiation. Data represent at least two independent experiments. *Significant increase in 3T3L1-G972R compared with 3T3L1-WT cells ($P<0.05$); **significant decrease in 3T3L1-G972R-PIO compared with 3T3L1-G972R cells ($P<0.05$).

at day 6 ($P<0.02$) and 4·8-fold at day 8 ($P<0.014$) (Fig. 7). These results suggest a possible involvement of Akt in the survival of the 3T3L1–G972R cells by inhibition of FKHRL1.

Activation of p42/p44 MAP kinases

To determine whether the G972R variant in the IRS-1 gene could also activate the pathway involving MAP kinases, we assayed the activation of p42/p44 MAP kinases during differentiation by immunoblotting cell lysates with antiphospho-p42/p44 MAP kinase antibody (Fig. 8A). Interestingly, in 3T3L1–G972R cells, phosphorylated (active) p42/p44 MAP kinases were lower by 1·6-fold at day 0 of differentiation ($P<0.012$), higher by 1·3-fold at day 2 ($P<0.03$) and higher by 1·8-fold at day 4 ($P<0.027$) than in 3T3L1–WT cells. To confirm the higher level of phosphorylated p42/p44 MAP kinases in 3T3L1–G972R cells, we performed a time–course experiment after insulin stimulation (100 nM 0–40 min). Immunoblot analysis showed that in 3T3L1–G972R cells the levels of p42/p44 MAP kinase phosphorylation remained higher by 3·8-fold ($P<0.003$), 4·7-fold ($P<0.013$) and 1·8-fold ($P<0.03$) after 5-, 10- and 20-min stimulation respectively than in 3T3L1–WT cells (Fig. 8B).

PIO enhances adipocyte differentiation and increases PPARγ expression in 3T3L1-G972R cells

Thiazolidinediones are known to promote adipocyte differentiation (Takamura *et al.* 2001) through PPARγ induction. We tested the hypothesis that PIO could reverse the impaired signaling and restore 3T3L1

adipocyte differentiation. Confluent 3T3L1–G972R pre-adipocytes were committed to adipocytes by exposing them to PIO in addition to insulin, isobutylmethyl-xanthine and dexamethasone. After induction, pre-adipocytes carrying the G972R variant accumulated fat, and a differentiated phenotype with more than 70% of the cells containing multilocular fat droplets could be observed by day 6 after induction (Fig. 1C). We then assessed the expression of PPARγ during adipocyte differentiation by immunoblotting and RT–PCR. Treatment of the 3T3L1-G972R with PIO enhanced PPARγ expression by 1·8-fold at day 2 of differentiation ($P=NS$), 2·5-fold ($P<0.012$) at day 4, 2·2-fold ($P<0.011$) at day 6 and 2·1-fold ($P<0.044$) at day 8 compared with untreated 3T3L1–G972R cells (Fig. 2C). We also assessed the expression of adipoQ and adipsin by RT–PCR. As expected, we observed an increase in the expression of all these markers in the 3T3L1–G972R cells treated with PIO compared with the untreated 3T3L1–G972R cells (data not shown).

PIO restores IRS-1 tyrosine phosphorylation, association of p85 with IRS-1, and PI 3-kinase activity in 3T3L1-G972R cells

Treatment of the 3T3L1 cells with 10 μM PIO, which has been shown to enhance insulin signaling (Iwata *et al.* 2001), does not cause a significant increase in amount of IRS-1 protein in the two cell lines but does cause an increase in IRS-1 tyrosine phosphorylation of 1·04-fold ($P=NS$) in 3T3L1–WT and 1·1-fold ($P=NS$) in 3T3L1-G972R cells (Fig. 3A). Binding of p85 subunit to IRS-1 is increased by 1·02-fold ($P=NS$) in 3T3L1–WT and by 1·2-fold ($P<0.03$) in 3T3L1–G972R cells. Thus, binding of p85 subunit to IRS-1 in 3T3L1–G972R cells is
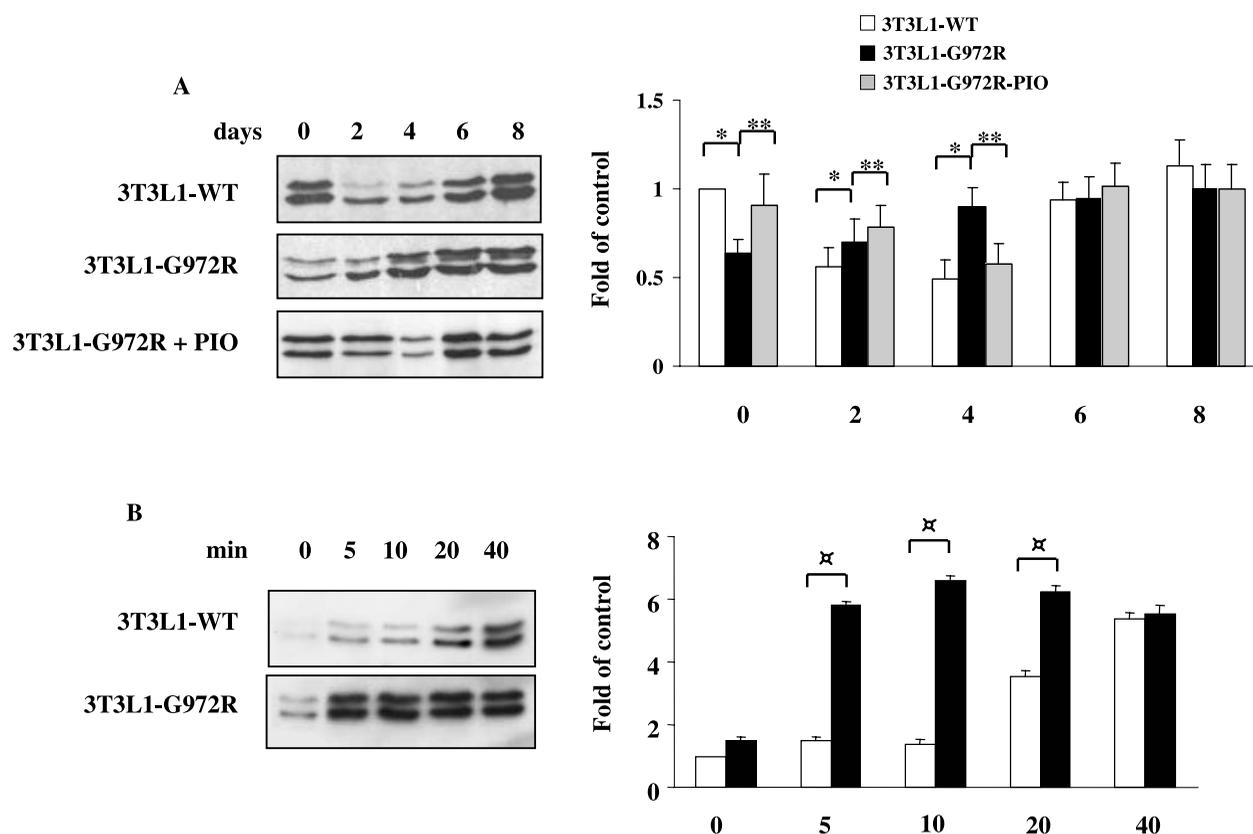
Figure 8 (A) p42/p44 MAP kinase phosphorylation during adipocyte differentiation in 3T3L1-WT, 3T3L1-G972R and 3T3L1-G972R-PIO cells. Protein lysates were immunoblotted with antiphospho-MAPKs antibody. PIO was added at 1 µM from the induction step of differentiation until full differentiation. Data represent three independent experiments. Time course (B) of insulin-stimulated (100 nM, 0–40 min) p42/p44 MAP kinase phosphorylation in confluent (day 0 of differentiation) 3T3L1 cells. Data from two separate experiments were calculated as fold of basal. ¤Significant increase in 3T3L1-G972R compared with 3T3L1-WT cells ($P<0.05$).

comparable to the insulin–stimulated association of p85 with IRS-1 in 3T3L1-WT cells (Fig. 3B). Similarly, PI 3-kinase activity was increased by 1·4-fold ($P<0.01$) in 3T3L1-WT and 1·3-fold ($P<0.04$) in 3T3L1-G972R cells (Fig. 4).

For better analysis of the direct effect of G972R IRS-1 in insulin signaling, we studied the effect of long-term PIO treatment in fully differentiated adipocytes. In particular, we have exposed fully differentiated 3T3L1-G972R–PIO cells to 10 µM PIO for an extra 48 h after day 8 of differentiation. The extra 48-h incubation were chosen in order to ensure complete disappearance of metabolically active PIO from medium after withdrawal. Results showed that IRS-1 phosphorylation and binding to PI 3-kinase were reduced respectively by 1·1-fold ($P<0.03$) and 1·9-fold ($P<0.002$) in cells without PIO compared with those where PIO was not withdrawn (Fig. 3C and D). This indicates that the G972R variant directly affects binding of IRS-1 to PI 3-kinase, since this defect is remedied by the presence of PIO.

Effect of PIO on IR tyrosine phosphorylation and interaction with IRS-1

After treatment with PIO, the binding of IRS-1 to the IR is increased by 1·5-fold ($P<0.01$) in 3T3L1-WT and 1·01-fold ($P=$NS) in 3T3L1-G972R cells compared with insulin-stimulated cells (Fig. 5A). Treatment of the 3T3L1 cells with PIO also causes an increase in IR tyrosine phosphorylation of 1·1-fold ($P=$NS) in 3T3L1-WT and 1·4-fold ($P<0.02$) in 3T3L1-G972R cells (Fig. 5B).

PIO restores both Ser[473] and Thr[308] phosphorylation of Akt

Treatment of the 3T3L1-G972R cells with PIO caused a reduction in both Thr[308] and Ser[473] phosphorylation of Akt during adipocyte differentiation, as in 3T3L1-WT cells (Fig. 6A and B). In comparison with 3T3L1-G972R cells, treatment of 3T3L1-G972R cells with PIO caused a decrease of 3·3-fold at day 6 of differentiation ($P<0.01$) and 3·5-fold at day 8 ($P<0.01$) in the phosphorylation
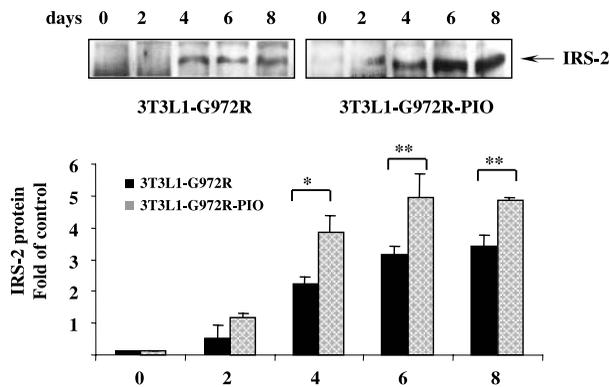
Figure 9 Effect of PIO on IRS-2 expression during adipocyte differentiation. Transfected 3T3L1 were incubated in presence or absence of 1 µM PIO for 8 days. Protein lysates were immunoblotted with monoclonal anti-IRS-2 antibody. Data represent at least two independent experiments. Significant increase in 3T3L1-G972R cells after PIO treatment is shown ($*P<0.05$; $**P<0.01$).

at Ser^{473}. The phosphorylation of Akt at Thr^{308} showed a pattern comparable to Ser^{473}.

In homology with Akt, FKHRL1 phosphorylation in 3T3L1-G972R cells with PIO caused a decrease of 1·6-fold at day 4 of differentiation ($P<0.04$), 3·7-fold at day 6 ($P<0.03$) and 6·1-fold at day 8 ($P<0.02$) (Fig. 7).

PIO restores p42/p44 MAP kinase phosphorylation

Treatment of the 3T3L1-G972R with PIO restored p42/p44 MAP kinase phosphorylation during adipocyte differentiation to levels similar to 3T3L1-WT cells (Fig. 8A). Compared with 3T3L1-G972R cells, 3T3L1-G972R-PIO cells showed an increase of 1·4-fold at day 0 of differentiation ($P<0.01$), a decrease of 1·2-fold at day 2 ($P<0.04$) and a decrease of 1·7-fold at day 4 ($P<0.008$) in the amount of phosphorylated (active) p42/p44 MAP kinases, results similar to those of 3T3L1–WT.

PIO increases IRS-2 expression

It has been recently demonstrated that PPARγ activation rapidly turns on the gene of the key signaling molecule IRS-2 (Smith *et al.* 2001), which is then phosphorylated in tyrosine and associates with p85, although less than IRS-1, in 3T3L1 cells (Inoue *et al.* 1998). Therefore, we analyzed the effect of PIO on IRS-2 expression in transfected 3T3L1 cells during adipocyte differentiation.

In untreated 3T3L1-G972R cells, IRS-2 expression is low at days 0 and 2 of differentiation and is modestly increased during the process of adipocyte differentiation (Fig. 9). Conversely, in 3T3L1-G972R cells treated with PIO, we observed a greater increase in the expression of IRS-2 gene. Thus, compared with untreated 3T3L1-G972R cells, IRS-2 expression in 3T3L1-G972R-PIO

cells was 1·7-fold higher at day 4 of differentiation ($P<0.045$), 1·8-fold at day 6 ($P<0.004$) and 1·6-fold at day 8 ($P<0.008$).

Discussion

IRS-1 is considered the major IRS in most insulin-sensitive tissue. Genetic analysis of the IRS-1 gene has revealed several base-pair changes that result in amino-acid substitution (Almind *et al.* 1993, Laakso *et al.* 1994, Hitman *et al.* 1995, Imay *et al.* 1997), and the most common of these is a glycine to arginine substitution at codon 972 (G972R), which has been shown to be strongly associated with obesity (Clausen *et al.* 1995, Baroni *et al.* 2001). Furthermore, higher insulin resistance is associated with the G972R variant in obese subjects, suggesting an interaction between the G972R mutation and obesity (Clausen *et al.* 1995, Baroni *et al.* 2001). These observations led us to carry out a functional study to assess whether there could be a direct impact of this genetic variant on insulin signaling in adipocytes.

We observed that 3T3L1 cells transfected with the G972R variant did not differentiate, thus suggesting that this variant affects insulin signaling during adipocyte differentiation. Indeed, in 3T3L1-G972R cells, the expression of the PPARγ gene was significantly reduced, together with a low level of expression of the specific adipocyte differentiation markers aP2, adiponectin and adipsin.

In the attempt to determine at which step the G972R variant could alter adipocyte differentiation, we examined several proteins involved in insulin signaling downstream of IRS-1. During activation of PI 3-kinase, the SH2 domains of p85 regulatory subunit bind directly to specific phosphorylated YMXM motifs present in the IRS-1 protein and consequently undergo the conformational changes required for the activation of the catalytic subunit p110 of the PI 3-kinase (Meyers *et al.* 1996). We found that, after insulin stimulation, the G972R variant in 3T3L1 cells caused a 17% decrease in IRS-1 phosphorylation. Furthermore, the 3T3L1-G972R cells exhibited 25% lower binding of p85 subunit to IRS-1 in response to insulin, in agreement with previous reports in other cell types (Almind *et al.* 1996, Hribal *et al.* 2000, Federici *et al.* 2001, McGettrick *et al.* 2004). We therefore determined the PI 3-kinase activity directly associated with IRS-1, and cells expressing the G972R variant of IRS-1 showed a 35% reduction in PI 3-kinase activity when compared with cells transfected with WT IRS-1. Recently, it has been demonstrated that PTEN, a phosphoinositide 3-phosphatase that, by metabolizing $PtdIns(3,4,5)P_3$, acts to suppress the PI 3-kinase/Akt pathway, plays a major role in antagonizing insulin signaling (Lo *et al.* 2004). The phosphorylation of the PTEN tail of three residues (S380, T382 and T383) regulates

protein stability and activity. We assessed the activity of PTEN by immunoblotting lysates from 3T3L1-WT and 3T3L1-G972R cells with anti-phospho-PTEN (Ser380) antibody, but found no difference in phosphorylation levels of PTEN between the two cell lines (data not shown).

A recent study showed that the presence of the G972R polymorphism markedly enhanced the association of the IRS-1 gene with the IR, allowing IRS-1 to act as an inhibitor of IR autophosphorylation (McGettrick *et al.* 2004). In order to determine the mechanism by which the phosphorylation of the G972R IRS-1 protein and its binding to p85 are reduced, we evaluated the association between IRS-1 and the IR. We observed that the interaction between the IR and the IRS-1 protein is enhanced in 3T3L1-G972R cells compared with 3T3L1-WT; in addition, the autophosphorylation of the IR is significantly inhibited.

Since PI 3-kinase is an upstream activator of Akt, we investigated whether the G972R variant alters Akt activation. Furthermore, since the activation of p42/p44 MAP kinases by insulin is PI 3-kinase/Ras dependent, we also investigated whether the decrease in p85 binding to G972R IRS-1 might influence p42/p44 MAP kinase signaling (Suga *et al.* 1997). Surprisingly, the IRS-1 G972R variant was found to be associated with a strong activation of Akt (in both Thr^{308} and Ser^{473} phosphorylated sites) and of p42/p44 MAP kinases compared with WT IRS-1. The higher activation of Akt in 3T3L1-G972R cells was not associated with higher activation of PDK1. It should be pointed out that Akt activation does not necessarily correlate with adipocyte differentiation, because both insulin and other growth factors, such as platelet-derived growth factor, activate Akt in 3T3L1 preadipocytes, but only insulin induces adipogenesis (Gagnon *et al.* 1999). Moreover, a few studies have observed that Akt may be activated by a PI 3-kinase-independent pathway (Kim *et al.* 1999, Venable *et al.* 2000, Nadler *et al.* 2001). Moreover, several studies have demonstrated that under certain circumstances (stress, growth factors, withdrawal, UV irradiation, matrix detachment, DNA damage or survival factors), Akt activity is both necessary and sufficient for cell survival and regulation of the apoptotic machinery (Chen *et al.* 1998, Biggs *et al.* 1999, Datta *et al.* 1999). In support of this hypothesis is the observation that Forkheads, which are potent activators of genes involved in apoptosis and are inactivated by Akt through phosphorylation, are strongly phosphorylated (thus inactive) in 3T3L1-G972R cells. Thus, the high levels of Akt and FKHRL1 phosphorylation that we observed in 3T3L1-G972R cells might be explained as a response to cellular stress due to impairment of insulin signaling.

An antagonistic relationship between p42/p44 MAP kinase activation and adipocytic differentiation has been described (Font de Mora *et al.* 1997), together with the observation that MAP kinase activation in 3T3L1 cells mediates signaling events that oppose differentiation, such as mitogenesis. It has been shown that upon reaching confluence (day 0), 3T3L1 cells undergo limited clonal expansion (days 1–2), postmitotic growth arrest (days 3–4) and terminal differentiation (days 4–10). The postmitotic growth arrest, referred to as G_D, has been shown to be required for terminal differentiation (Delmas *et al.* 2001). Interestingly, in 3T3L1-G972R, we observed at days 2 and 4 (days of commitment of 3T3L1 differentiation) an increase in p42/p44 MAP kinase phosphorylation compared with 3T3L1-WT. This observation suggests that, since insulin signaling is impaired by the G972R variant, the Ras/ERK pathway is activated, stimulating proliferation instead of differentiation.

In summary, we observed that the G972R variant of the IRS-1 gene impairs insulin signaling and adipocyte differentiation *in vitro*, and it appears to exert its action in a negative-dominant-like manner. Thus, we studied whether the treatment with PPARγ agonists, which have been shown to promote adipocyte differentiation and to improve insulin sensitivity by upregulating PPARγ mRNA levels (Takamura *et al.* 2001), could modify the negative effects of the G972R variant.

In rats, it has been shown that treatment with PIO increases the number of small adipocytes (which are fully responsive to insulin), together with the reduction of existing large adipocytes (de Souza *et al.* 2001). Different mechanisms have been proposed on how a receptor expressed predominantly in fat tissue may improve insulin sensitivity in all of the major insulin-sensitive tissues. It has been suggested (Smith 2002) that improvement of insulin sensitivity may be explained by the endocrine function of adipose tissue, where PPARγ agonists, by restoring adipocyte physiology, can improve the release of adipokines that are known to modulate insulin action, such as adiponectin.

Treatment of the 3T3L1-G972R with PIO enhanced PPARγ expression and restored adipocyte differentiation. Phosphorylation of Akt and p42/p44 MAP kinases returned to levels comparable to 3T3L1-WT cells. Adiponectin mRNA was also restored to normal levels. Miyazaki *et al.* (2003) have shown *in vivo* that rosiglitazone treatment enhances downstream insulin-receptor signaling in muscle. In particular, IRS-1 tyrosine phosphorylation and p85 binding are significantly improved, an effect similar to what we observed *in vitro*.

This was particularly evident in fully differentiated 3T3L1-G972R-PIO cells exposed to long-term PIO incubation, where we observed that IRS-1 binding to PI 3-kinase was maintained in cells with PIO compared with those where PIO was withdrawn, thus indicating that the G972R variant directly affects binding of IRS-1 to PI 3-kinase. Moreover, previous studies have shown that treatment of 3T3L1 adipocytes with PIO alone increased both protein levels and insulin-stimulated tyrosine

phosphorylation of IR and IRS–1 (Iwata *et al.* 2001). Association of p85 with IRS–1 and PI 3-kinase activity were also increased by PIO (Iwata *et al.* 2001). Since PPARγ has been reported to cross–regulate C/EBPα, and the regulatory regions in IR and IRS–1 genes were reported to contain C/EBP-binding sequence (Wu *et al.* 1999), the activation of PPARγ by PIO may affect expression of C/EBPα, which in turn increases expression of IR and IRS–1. In fact, in our study, we observed that treatment with PIO of 3T3L1-G972R cells caused an increase in the amount of IRS–1 protein (although not statistically significantly). We can speculate that this increase helps to improve the IRS–1/PI 3-kinase pathway and activity.

Furthermore, Smith *et al.* (2001) demonstrated that, in both differentiated 3T3L1 and human adipose tissue, there is a specific activation of the IRS–2 gene by PPARγ agonists, without change in IRS–1, suggesting that this is one of the mechanisms of the insulin–sensitizing effect of these drugs. Previous studies have shown that the different localization of IRS–1 and IRS–2 may explain why IRS–2 contributes less to tyrosine phosphorylation of IRS proteins and p85 association than IRS–1 in 3T3L1 adipocytes. Tyrosine phosphorylation of IRS–1 declines slowly over 10 min after insulin stimulation, while IRS–2 phosphorylation declines more rapidly (Inoue *et al.* 1998).

In our study, treatment with PIO of 3T3L1-G972R cells caused an increase in the expression of IRS–2 gene, compared with untreated 3T3L1-G972R, and a concomitant normalization of the insulin–induced signaling pathway, overcoming the block caused by the presence of the G972R variant. Thus, it seems likely that the increase in both IRS–1 and IRS–2 expression helps to ameliorate the insulin signaling in these cells. Finally, the increase in IR autophosphorylation induced by PIO, probably through the increase in IR and IRS–1/IRS–2 expression, may be another mechanism by which PIO may improve insulin action.

Previous studies have shown that the G972R variant of the IRS–1 gene is strongly associated with insulin resistance in obesity (Clausen *et al.* 1995, Baroni *et al.* 2001). It seems that this variant *in vivo* does not affect adipocyte differentiation but may impair insulin action. Control of preadipocyte to adipocyte progression is crucial in regulating energy storage and release. In complex organisms with balanced energy homeostasis, only a portion of the preadipocyte pool become differentiated adipocytes; the rest of the preadipocytes remain quiescent. A complex pattern of factors (including food intake, hormonal stimulation, growth factors, cytokines with opposite effects and fat cell size) interact to maintain an adequate number of small differentiated adipocytes, which are fully responsive to insulin. It should also be noted that adipose tissue contains not just mature adipocytes, but a variety of cell types, including fibroblasts, endothelial cells and preadipocytes at various stages of development, all contributing to the local milieu that regulates adipogenesis. The loss of differentiation *in vitro* could be explained as inability to reproduce the same conditions as *in vivo,* where several cofactors act to stimulate adipocyte differentiation. Thus, *in vivo*, the impairment of insulin signaling determined by the G972R variant may cause insulin resistance without affecting adipocyte differentiation.

In conclusion, we have demonstrated that the G972R variant impairs insulin action in 3T3L1 cells. Impairment of insulin signaling by the G972R variant of IRS–1 has also been demonstrated in other cell types, such as L6 miocytes (Hribal *et al.* 2000), human pancreatic islets (Federici *et al.* 2001) and myeloid progenitor 32D(IR) (Almind *et al.* 1996). Furthermore, we have shown that the G972R variant binds with higher affinity to IR, thus reducing IR autophosphorylation and inhibiting insulin signaling, as described previously by Kahn and coworkers (McGettrick *et al.* 2004).

Taken together, these observations indicate that this IRS–1 gene variant affects insulin action, and support the hypothesis that this variant is involved in the pathogenesis of insulin resistance. We have also shown that treatment with PPARγ agonists restores normal 3T3L1 adipocyte differentiation and insulin signaling. Subjects carrying this variant have been shown to be more insulin resistant, particularly when obesity is present. If confirmed *in vivo*, these observations may have application in the treatment of insulin resistance in genetically predisposed obese subjects.

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