Diabetologia (2000) 43: 718–722

Diabetologia

Short Communication

Adenovirus-mediated expression of a naturally occurring Asp905Tyr variant of the glycogen-associated regulatory subunit of protein phosphatase-1 in L6 myotubes

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Abstract

Aims/hypothesis. The glycogen-associated protein phosphatase-1 (PP1G) is thought to play an important part in the regulation of skeletal muscle glycogen content. We have previously identified an Asp905Tyr polymorphism of the glycogen-associated regulatory subunit of the protein phosphatase 1 (*PPP1R3*) gene which among healthy subjects was associated with decreased insulin stimulated non-oxidative glucose metabolism, i.e. primary glycogen synthesis. In this study, the functional effect of the polymorphism was examined in vitro.
Methods. Wild type (PPP1R3-Asp905) and mutant (PPP1R3-Tyr905) *PPP1R3* were expressed in L6 myotubes using adenovirus-mediated gene transfer. Basal and insulin-stimulated glucose uptake and glycogen synthesis were measured. Furthermore, the sensitivity of glycogen synthesis to a cyclic AMP agonist was measured.
Results. Compared with green fluorescent protein-transduced myotubes and non-transduced myotubes, overexpression of PPP1R3-Asp905 and PPP1R3-Tyr905 increased both basal and insulin-stimulated glycogen synthesis approximately twofold. Treatment of both non-transduced and *PPP1R3*-transduced L6 myotubes with a cAMP agonist decreased both basal and insulin-stimulated glycogen synthesis by about 40%. Overexpression of *PPP1R3* did not affect either basal or insulin-stimulated 2-deoxy-D-glucose uptake compared with green fluorescent protein-transduced cells.
Conclusion/interpretation. Results obtained from L6 myotubes transduced with PPP1R3-Asp905 or PPP1R3-Tyr905 showed no statistically significant difference. Therefore, the Asp905Tyr variant alone is unlikely to account for the decreased insulin stimulated non-oxidative glucose metabolism observed in the human study reported previously. [Diabetologia (2000) 43: 718–722]

Keywords *PPP1R3*, PP1G, codon 905 polymorphism, Type II diabetes, glycogen synthesis, non-oxidative glucose metabolism, L6 myotubes, adenovirus.

Received: 25 November 1999 and in revised form: 26 January 2000

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Abbreviations: PP1G, Glycogen associated protein phosphatase 1; *PPP1R3*, glycogen associated regulatory subunit of protein phosphatase 1; PP1C, catalytic subunit of protein phosphatase 1; I-1, inhibitor 1; cAMP, cyclic AMP; pfu, plaque-forming units; 2-DOG, 2-deoxy glucose; GFP, green fluorescent protein; WT, wild type; α-MEM, α-minimal essential medium; β-gal, beta-galactosidase.

Decreased insulin stimulated non-oxidative glucose disposal, which primarily reflects glycogen synthesis in skeletal muscle, is an early metabolic abnormality in patients with Type II (non-insulin-dependent) diabetes mellitus [1]. The glycogen-associated protein phosphatase 1 (PP1G), which is a holoenzyme composed of a regulatory subunit (G-subunit) and a catalytic subunit (PP1C), is a regulator of glycogen-bound substrates such as glycogen synthase and glycogen phosphorylase. It has been suggested that PP1G has an important role in hormonal regulation of the glycogen content in skeletal muscle [2]. Insulin is reported to stimulate PP1G activity through phosphoryla-

tion of Ser46 (site 1) on the G-subunit. This has been shown to enhance the phosphatase activity towards glycogen synthase and glycogen phosphorylase, thereby increasing glycogen synthesis and decreasing glycogenolysis [2]. In addition, Ser65 (site 2) of the G-subunits is phosphorylated in response to adrenaline, a β-adrenergic agonist that stimulates glycogen breakdown [2]. Phosphorylation of Ser65 releases PP1C from the G-subunit, making it more sensitive to the cytosolic inhibitor-1 protein (I-1) and decreasing glycogen synthesis and increasing glycogenolysis [2].

In insulin-resistant Pima Indians a reduced basal and insulin-stimulated activity of PP1G in skeletal muscle has been reported [3] and this could explain the decreased activation of glycogen synthase and subsequently the impaired insulin-stimulated glycogen synthesis observed in these patients. Altogether, these data indicate that the gene encoding the G-subunit, *PPP1R3* is a candidate gene for insulin resistance and Type II diabetes. We previously did a mutational analysis of the human *PPP1R3* gene among Type II diabetic patients and identified a common Asp905Tyr polymorphism [1]. The polymorphism was not associated with Type II diabetes or decreased insulin-stimulated, whole-body glucose uptake. A euglycaemic hyperinsulinaemic clamp done in combination with indirect calorimetry among healthy subjects indicated, however, that heterozygous carriers of the Tyr905 polymorphism had decreased insulin-stimulated non-oxidative glucose metabolism of peripheral tissues and increased basal glucose oxidation rate compared with non-carriers. In contrast, among the Pima Indians, the Asp905 allele was associated with reduced insulin-stimulated, whole-body glucose disposal under a euglycaemic hyperinsulinaemic clamp [4]. Among the Pima Indians the Asp905 allele is always coupled, however, to two other polymorphisms (Arg883 and ARE2). It is therefore uncertain whether the association is caused by the Asp905 allele. In the current study we addressed whether the Asp905Tyr variant has any functional effect on glycogen metabolism in in vitro experiments. We investigated the Asp905Tyr variant by using recombinant adenovirus transduction of L6 myotubes and Chinese hamster ovary cells overexpressing the insulin receptor (CHO-IR). These cells were transduced transiently with recombinant adenoviral constructs expressing PPP1R3-Asp905 and PPP1R3-Tyr905 to compare their role on basal and insulin-stimulated glucose uptake and glycogen synthesis. Furthermore, because PP1G is also involved in the adrenergic glycogenolytic pathway, we examined the responsiveness to a cAMP agonist.

Materials and methods

Materials. The L6 and CHO-IR cells were kindly provided by Dr A. Klip (The Hospital of Sick Children, Toronto, Canada) and Dr M. F. White (Joslin Diabetes Center, Harvard Medical School, Boston, USA), respectively. Plasmids pACCMV.pLpA, pJM17 and β-gal adenovirus were kindly provided by Dr. C. Newgard, University of Texas, Southwestern Medical Center, Dallas, USA. Sheep polyclonal anti-PPP1R3 was generated against human GST-PPP1R3 (1–243) and rabbit PPP1R3-PP1c complex and affinity purified against human GST-PPP1R3 (1–243). Horseradish peroxidase-conjugated, anti-sheep IgG was obtained from Rockland (Gilbertsville, Pa., USA). Cell culture reagents were purchased from Life Technologies (Rockville, Md., USA). All other reagents and chemicals were purchased from Sigma (St. Louis, Mo., USA).

Cell culture and transfections. The L6 cells were grown and maintained in α-minimal essential medium (α-MEM) containing 5 mmol/l glucose in the presence of 10 % fetal bovine serum and 1 % antimycotic-antibiotic solution (final concentrations, 100 U/ml penicillin G, 100 mg/ml streptomycin, and 250 ng/ml amphotericin-B) at 37 °C in an atmosphere of 5 % CO_2. The cells were allowed to grow to confluence and differentiation was induced by addition of differentiation medium (α-MEM supplemented with 2 % fetal bovine serum and 1 % antimycotic-antibiotic solution). Cells were used 4–6 days after induction of differentiation. Cell fusion was monitored by phase-contrast microscopy and only wells where more than 80 % of total cells were fused were used for transduction experiments. We grew 293 cells in DMEM with 10 % fetal bovine serum, 50 U/ml penicillin G, 50 mg/ml streptomycin at 37 °C, 5 % CO_2. The CHO-IR cells were maintained in culture in Ham's F-12 media, supplemented with 10 % fetal bovine serum, 50 U/ml penicillin G, 50 mg/ml streptomycin at 37 °C, 5 % CO_2.

Plasmids. A full-length human *PPP1R3* cDNA was prepared by PCR using non-overlapping segments of human *PPP1R3* [5] as template and extended primers to create an overlap. This was followed by a second round of PCR with the outermost primers to create the full length cDNA. The *PPP1R3* was subcloned into the *Sal*I and *Hind*III sites of pACCMV.pLpA [6] to generate wild-type (WT) pACCMV.pLpA-PPP1R3-WT. Site-directed mutagenesis to create the Asp905Tyr variant was carried out using recombinant PCR. Generally, PCR was done with *Pfu* polymerase and the numbers of cycles were kept low (typically less than 20 cycles) to reduce the risk for PCR misincorporations. The *GFP* gene was subcloned from pEGFP-1 (Clontech) into the *Bam*H1 and *Xba*I of pACCMV.pLpA to generate pACCMV.pLpA-GFP. All cloned PCR segments were sequenced to verify the fidelity of the PCR.

Generation of recombinant adenoviruses and transduction of L6 myotubes and CHO-IR cells. Recombinant adenoviruses were generated fundamentally as described previously [6, 7] but the preparations of adenovirus were purified by a double cesium chloride centrifugation procedure instead of only one cesium chloride centrifugation. The titre of the recombinant adenovirus was determined by limiting dilution and plating by agar overlay. Plaque forming units (pfu) were counted and expressed as pfu/ml. Stocks (5×10^{10} pfu/ml) of recombinant virus were prepared by dilution of the virus in the relevant cell culture medium. A PCR-RFLP was carried out as described [4] with purified diluted virus as template to confirm the genotype of the recombinant adenovirus. Transduction of L6 myotubes

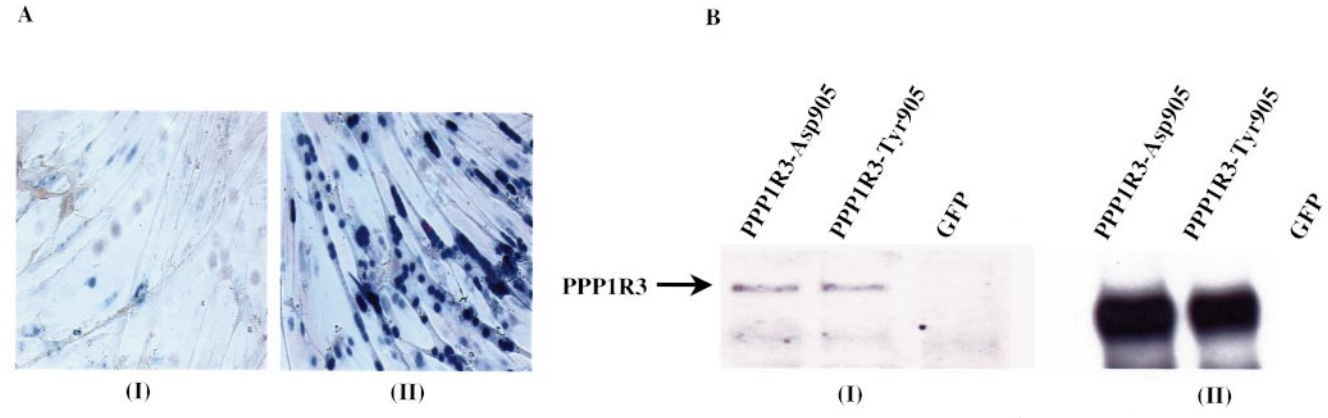

Fig. 1. A L6 myotubes were transduced for 2 h (I) or overnight (II) with 5×10^8 and 5×10^9 pfu/ml recombinant β-gal encoding adenovirus, respectively. After an additional 24 h in fresh medium the myotubes were stained for β-gal using standard procedures. β-gal translocates to the nucleus due to a nuclear localisation signal and each blue spot represents a nucleus in the multinucleated myotube. **B** Expression of PPP1R3-Asp905, PPP1R3-Tyr905, and green fluorescent protein (GFP) in transduced L6 myotubes. We analysed 40 mg of total protein for expression using anti-PPP1R3 antibodies generated against human (I) and rabbit (II) PPP1R3 sequences

(5×10^9 pfu/ml) and CHO-IR (1×10^9 pfu/ml) cells was done overnight with agitation at 37 °C, 5 % CO_2 and experiments were done after an additional 24-h incubation in fresh medium.

Glucose transport. Cells cultured in 24-well plates were incubated in serum-free α-MEM for 4 h, washed with glucose-free MEM and incubated for 30 min with or without 100 nmol/l insulin in 500 µl glucose-free MEM. We added 100 µmol/l 2-[^{3}H]deoxy-D-glucose for 10 min. Cells were washed three times with ice-cold PBS and lysed in 300 µl 1 mol/l NaOH. After 30 min at 37 °C 37.5 µl 12.1 mol/l HCl was added to the lysate and aliquots were used for liquid scintillation counting and determination of protein content.

Glycogen synthesis. Cells in 24-well plates were deprived of serum for 4 h at 37 °C in Krebs-Ringer-bicarbonate-HEPES (KRBH) containing 5 mmol/l glucose (2.5 mmol/l for CHO-IR cells) and 0.5 % BSA followed by incubation in KRBH:0.5 % BSA for 15 min. Cells were treated either with water or 100 nmol/l insulin (final concentration) for 15 min and subsequently incubated with 5 mmol/l D-[U-^{14} C]glucose (7.4 kBq) and in some experiments with 0.1 mmol/l dibutyryl cAMP for 60 min. Cells were washed three times with ice-cold PBS, rendered soluble in 250 µl 30 % (w/v) KOH and the lysates were transferred to tubes containing 5 µl 100 mg/ml carrier glycogen. The mixtures were boiled 30 min in a water bath and subsequently spotted on pieces of 3MM Watmann filter papers that were immersed in ice-cold 70 % ethanol. The filters were washed three times (30 min each), dried by washing in acetone and the radioactivity measured by a scintillation counting.

Glycogen content. The L6 myotubes were cultured in 6-well plates in DMEM (25 mmol/l glucose) and were treated either with water or 100 nmol/l insulin (final concentration) for 90 min. Subsequently cells were washed five times with ice-cold PBS and the glycogen content was determined after acid hydrolysis by a standard hexokinase method.

Immunoblotting. Cell lysates were subjected to polyacrylamide gel electrophoresis and immunblotting as reported [7] using 6 % minigels. Membranes were incubated overnight with the anti-PPP1R3 antibody (0.5 µg/ml and 0.1 µg/ml in final concentrations for antibodies generated against rabbit and human *PPP1R3* cDNA, respectively). A horseradish peroxidase anti-sheep IgG was used as secondary antibody. Proteins were visualised by enhanced chemiluminescence.

Statistical analysis. Data are presented as mean ± SEM. We used ANOVA to test for significant differences between the groups using Statistical Package of Social Science for Windows (SPSS), version 9.0. We considered p less than 0.05 to be significant.

Results

The transduction efficiency was assessed by transducing cells with beta-galactosidase (β-gal) encoding recombinant adenovirus. More than 90 % of CHO-IR cells (transduced overnight with 1×10^9 pfu/ml) and L6 myotubes (transduced overnight with 5×10^9 pfu/ml) had blue nuclei (Fig. 1A; II) but cells transduced with only 5×10^8 pfu/ml virus for 2 h had very little blue colour in the presence of the β-gal chromogenic substrate (Fig. 1A; I). Transduction with PPP1R3-Asp905, PPP1R3-Tyr905, β-gal and GFP resulted in no apparent differences in the morphological features of L6 myotubes and CHO-IR cells.

Polymerase chain reaction-RFLP using recombinant adenovirus stocks as template verified that PPP1R3-Tyr905 was not contaminated with PPP1R3-Asp905 or vice versa (data not shown). To confirm that transduction with recombinant adenovirus encoding PPP1R3-Asp905 and PPP1R3-Tyr905 resulted in successful and similar expression we immunoblotted L6 myotube lysates with anti-PPP1R3 antibody. Figure 1B shows representative western blots using anti-PPP1R3 antibody (generated against (I) human and (II) rabbit *PPP1R3* sequence, respectively) confirming a similar expression level of the two constructs.

Overexpression of PPP1R3-Asp905 and PPP1R3-Tyr905 in L6 myotubes led to a significant increase

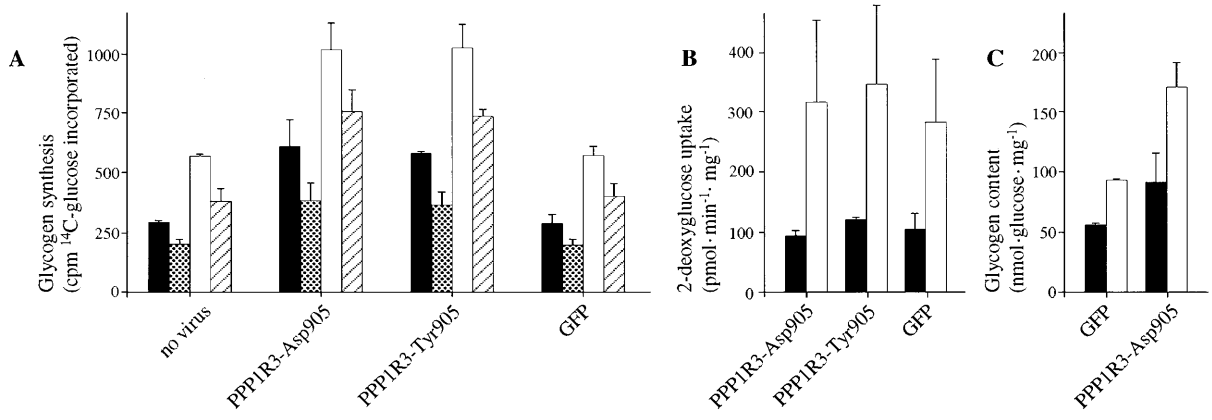

Fig. 2 A–C. Glycogen synthesis (**A**), 2-DOG uptake (**B**) and glycogen content (**C**) in L6 myotubes expressing PPP1R3-Asp905, PPP1R3-Tyr905 or green fluorescent protein (GFP). L6 myotubes were transduced overnight with 5×10^9 pfu/ml of the indicated recombinant adenovirus and after an additional 24 h in fresh medium the experiments were done as described in Materials and methods. The results shown are representative of two to three independent experiments, each done in triplicate and data are presented as means ± SEM. **A** For each group; insulin was different from basal, dibutyryl cAMP different from basal, and insulin + dibutyryl cAMP different from insulin at $p < 0.01$. no insulin, no insulin + dibutyryl cAMP, + insulin and + insulin + dibutyryl cAMP were higher in PPP1R3-Asp905/PPP1R3Tyr905 compared with no virus/GFP, $p < 0.01$. **B** For each group insulin was different from basal, $p < 0.01$. **C** Basal different from insulin and PPP1R3-Asp905 different from GFP at basal and insulin, $p < 0.01$. ■ no insulin; ▦ no insulin, + 0.1 mmol/l dibutyryl cAMP; □, + 100 nmol/l insulin; ▨ + 100 nmol/l insulin, + 0.1 mmol/l dibutyryl cAMP

in basal and insulin-stimulated glycogen synthesis (approximately twofold) measured as the incorporation of D-[U-^{14}C]glucose into glycogen compared with non-transduced or *GFP* expressants (Fig. 2A). The fold stimulation by insulin did not differ statistically significantly between cells transduced with *PPP1R3* and non-transduced/*GFP*-transduced cells but the increment above basal was greater in *PPP1R3*-expressing cells. Treatment with dibutyryl cAMP decreased basal and insulin-stimulated glycogen synthesis by approximately 40 % in both *PPP1R3* and non-transduced/*GFP*-transduced cells. Importantly, no statistically significant differences in responsiveness to insulin or dibutyryl cAMP were observed in L6 myotubes expressing PPP1R3-Asp905 and PPP1R3-Tyr905, respectively.

To assess insulin signalling to glucose transport, experiments assessing uptake of 2-deoxy glucose (2-DOG) were carried out in transduced myotubes. They showed that overexpression of PPP1R3-Asp905 or PPP1R3-Tyr905 did not alter the glucose uptake compared with *GFP*-transduced L6 myotubes (Fig. 2B). To determine whether expression of *PPP1R3* actually results in increased glycogen we measured the glycogen content in L6 myotubes. The glycogen content in L6 myotubes transduced with PPP1R3-Asp905 was increased approximately 80 % in the basal state and after insulin stimulation compared with myotubes transduced with *GFP* adenovirus (Fig. 2C).

To confirm the results, glycogen synthesis and 2-DOG experiments were also carried out in CHO-IR cells transduced with PPP1R3-Asp905 and PPP1R3-Tyr905. The results were similar to those obtained from L6 myotubes but the increases in both basal and insulin-stimulated glycogen synthesis was even higher (approximately sixfold). Glycogen synthesis (cpm per well, means ± SEM of two experiments in triplicate) in basal and insulin-stimulated states were, respectively; non-transduced cells (166 ± 15 and 278 ± 12), PPP1R3-Asp905 (1139 ± 82 and 1660 ± 223) and PPP1R3-Tyr905 (1156 ± 82 and 1715 ± 223). The overexpression did not have any effect on 2-DOG uptake (data not shown) and there were no statistically significant differences between CHO-IR cells transduced with PPP1R3-Asp905 and PPP1R3-Tyr905.

Discussion

We investigated the functional effect of a naturally occurring polymorphism of *PPP1R3* (Asp905Tyr) by overexpression experiments in L6 myotubes and CHO-IR cells. We were not able to detect any significant differences between cells transduced with wild-type or mutant *PPP1R3* for basal or insulin-stimulated glycogen synthesis or 2-DOG uptake in either of the cell lines. Overexpression of PPP1R3-Asp905 or PPP1R3-Tyr905 resulted in increased basal and insulin-stimulated glycogen synthesis but had no effect on glucose uptake. Furthermore, L6 myotubes expressing PPP1R3-Asp905 or PPP1R3-Tyr905 showed no differences in cellular responsiveness to cAMP agonist for basal and insulin-stimulated glycogen synthesis. These results do not agree with recent studies examining L6 clones transfected stably with flag-tagged PPP1R3-Asp905 or PPP1R3-

Tyr905 [8, 9]. The main conclusions from those studies were: overexpression of *PPP1R3* results in an increased insulin-stimulated glycogen synthesis whereas the basal levels are unaffected and PPP1R3-Tyr905 has an increased sensitivity towards cAMP agonists compared with PPP1R3-Asp905. The reasons for the discrepancies are not known but there are several possible explanations. Firstly, the methodology used differs because we have used a transient transfection procedure that efficiently transfects a whole population of cells, whereas these studies [8, 9] used stable constitutive expression of *PPP1R3*, in which one clone from each construct was examined. Another reason could be that we used a human *PPP1R3* clone whereas the other studies used a clone derived from rabbit cDNA. The amino acid homology between human and rabbit is 73 % [5] and although this is a relatively high homology the functional effect of sequence variants might be species specific.

Another major difference between our data and results previously reported [8, 9] is that a statistically significantly increased insulin-stimulated 2-DOG uptake was found in clones stably overexpressing *PPP1R3* [8]. In contrast, our data showed that overexpression of *PPP1R3* did not have any effect on either basal or insulin-stimulated glucose uptake. Similar results have been reported from transgenic mice overexpressing or deficient in *PPP1R3* [10] that do not have altered basal or insulin-stimulated glucose uptake in soleus muscles. These results together with our data challenge the suggested mechanism of *PPP1R3* and indicate that *PPP1R3* is involved in the regulation of glycogen synthesis but it might not be a major protein involved in insulin-stimulated activation of glycogen synthesis in skeletal muscle.

In conclusion, L6 myotubes and CHO-IR cells transduced with PPP1R3-Tyr905 or PPP1R3-Asp905 showed increased glycogen synthesis and glycogen content compared with control cells. There were, however, no statistically significant differences in basal or insulin-stimulated glucose uptake or glycogen synthesis between the two *PPP1R3* constructs. Furthermore, L6 myotubes transduced with PPP1R3-Tyr905 or PPP1R3-Asp905 did not have altered responsiveness to a cAMP agonist.

Acknowledgements. The study was supported by grants from the Danish Medical Research Council, the Danish Research Academy, the Danish Diabetes Association, the Velux Foundation and EEC (BMH4-CT95–0662 and BMH4-CT98–3084), NIH DK R01–43051 and AG00294, and the UK Medical Research Council. The authors thank H. Fjordvang and E. Hadro for technical assistance and G. Lademann for secretarial support. We thank Dr. C. Newgard for plasmids, Dr. M. White and Dr. A. Klip for cells.

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