Expression of the splice variants of the p85 α regulatory subunit of phosphoinositide 3-kinase in muscle and adipose tissue of healthy subjects and type 2 diabetic patients

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The regulation by insulin of the expression of the p85 α regulatory subunit of phosphoinositide 3-kinase (PI 3-kinase) is impaired in skeletal muscle and adipose tissue of type 2 diabetic patients. The gene encoding p85 α (named *grb-1*) can generate several variants by alternative splicing, all being able to activate the p110 catalytic subunits of PI 3-kinase. Our aims were (i) to determine the mRNA expression profiles of these variants in human skeletal muscle and adipose tissue; (ii) to investigate the effect of insulin on their expression *in vivo* and *in vitro* in muscle and (iii) to verify whether this regulation is defective in type 2 diabetes. We determined the human genomic organization of *grb-1* and set up reverse transcriptase competitive PCR assays for the quantification of each mRNA variant. In muscle, p85 α and p50 α mRNAs were the most abundant, and p55 α represented less than 20% of all *grb-1*-derived mRNAs. In adipose tissue, p85 α was

expressed predominantly and p55 α mRNA was not detectable. These expression profiles were not different in type 2 diabetics. During a 3 h hyperinsulinaemic clamp, insulin increased the mRNA expression of the three variants in muscle of control subjects. In diabetic patients, the effect of insulin on p85 α and p50 α mRNAs was blunted, and largely reduced on p55 α transcripts. In cultured human myotubes, up-regulation of p85 α , p55 α and p50 α mRNAs by insulin was abolished by LY294002 (10 μ M) and by rapamycin (50 nM), suggesting that the PI 3-kinase/protein kinase B/p70 S6 kinase pathway could be involved in the stimulation of *grb-1* gene expression by insulin in human muscle cells.

Key words: *grb-1*, insulin signalling, myotubes, quantitative PCR.

INTRODUCTION

Class IA phosphoinositide 3-kinases (PI 3-kinases) are lipid kinases that catalyse the specific phosphorylation of PtdIns, PtdIns(4,5) P_2 at position D-3 of the inositol ring. PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 are important second messengers that regulate diverse cellular functions and that are implicated in the biological responses of a variety of growth factors and hormones, including insulin [1–3]. Indeed, a large body of evidence clearly demonstrates that the stimulation of PI 3-kinase activity plays a crucial role in insulin action, especially on glucose transport, glycogen synthesis and anti-lipolysis [4]. Moreover, the PI 3-kinase signal-transduction pathway appears to be impaired in skeletal muscle of insulin-resistant patients, such as those suffering from type 2 diabetes mellitus [5,6] or obesity [7].

PI 3-kinases are heterodimers composed of a p110 catalytic subunit and a regulatory subunit. Four isoforms of the p110 catalytic subunits (α , β , γ and δ), derived from different genes, have been reported so far [8–11]. The p110 α and p110 β are thought to be the main isoforms involved in insulin action in peripheral tissues [4] and are expressed in insulin-sensitive tissues in humans [12,13]. In contrast, p110 δ is predominantly expressed in leucocytes [11] and activation of p110 γ is not dependent on the regulatory subunits that normally participate in insulin signalling [10]. The regulatory subunits act as adapter molecules to activate the p110 catalytic subunits of PI 3-kinase by virtue of the interaction of their SH2 (Src homology 2) domains with specific phosphorylated tyrosine residues of upstream signalling

molecules [1,3]. Among these molecules, several tyrosine kinase receptors as well as the different insulin receptor substrates (IRSs) can interact with the regulatory subunits of PI 3-kinases [1,3]. To date, at least five regulatory subunits have been identified in mammals. The p85 α , p55 α and p50 α isoforms are generated from the same gene by alternative splicing and, possibly, by different promoter usage [14]. The p85 α protein is composed of a number of modular domains, including two SH2 domains located in the centre (amino acids 333–430) and at the C-terminus (amino acids 624–720) of the molecule that bind to specific phosphotyrosines of IRS-1 and IRS-2 [1]. These SH2 domains are preserved in the p55 α and p50 α splice variants [15,16]. The p85 β regulatory subunit is similar in structure to p85 α with about 62% identity at the amino acid level [17]. Initial reports indicated that p85 β is primarily expressed in brain and in lymphoid tissues [18] and that it does not transduce the insulin signal in transfection experiments [19]. More recent works suggest however that p85 β may play a role in insulin action in skeletal muscle [20] and in heart [21]. Finally, another regulatory subunit, termed p55 γ or p55^{PIK}, has been described, presenting about 70% amino acid identity with the two SH2 domains and the inter-SH2 region of the p85 α gene [22]. This molecule is the product of a separate gene, and appears to be more abundant in brain and testes [22].

Transgenic mice with a disrupted p85 α PI 3-kinase gene (*pi3kr1*) are characterized by increased insulin sensitivity and hypoglycaemia [23,24], demonstrating that the regulatory subunits generated from this gene have a role in glucose homeostasis and in insulin action *in vivo*. Although unexpected, the observed

Abbreviations used: PI 3-kinase, phosphoinositide 3-kinase; IRS, insulin receptor substrate; RT-cPCR, reverse transcriptase competitive PCR; SH2 domain, Src homology 2 domain.

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increase in insulin action could be a consequence of compensatory up-regulation of the p50 α splice variant in mice lacking the p85 α molecule only [23] or of increased expression of the p85 β and p55 γ regulatory subunits in mice lacking all isoforms derived from *pi3kr1* [24]. These studies illustrated the complexity of the regulatory network at the level of PI 3-kinase in insulin action.

In humans, it has been clearly demonstrated that skeletal-muscle insulin resistance in type 2 diabetes involves impaired insulin signalling through the PI 3-kinase pathway [5,6,25]. We have recently reported that the steady-state mRNA expression levels of the p85 α regulatory subunit and the p110 α and p110 β catalytic subunits is not altered in the skeletal muscle of type 2 diabetic patients [13]. However, little is known regarding the expression of the other subunits of the PI 3-kinases in human muscle and possible alterations during type 2 diabetes mellitus. By screening a human muscle cDNA library, Antonetti et al. [15] identified new alternatively spliced forms of p85 α and p50 α that contain an insert of eight amino acid residues in the inter-SH2 region. This insertion results in the addition of two potential serine phosphorylation sites in the vicinity of the previously known serine (Ser-608) autophosphorylation site. Using a unique model of human muscle-strip preparation, it has been shown that human muscle expresses the five PI 3-kinase regulatory subunits described so far (p85 α , p55 α , p50 α , p85 β and p55 γ) as well as two other proteins derived from the p85 α gene [20] that may eventually correspond to the forms containing the insert described by Antonetti et al. However, up to now, the relative expression of these different forms of regulatory subunit has not been characterized in the insulin-sensitive tissues of healthy subjects and type 2 diabetic patients.

In addition, although we have observed that the mRNA expression level of the p85 α regulatory subunit is not altered in skeletal muscle and adipose tissue of type 2 diabetic patients [13,26], we have found that the short-term regulation by insulin of p85 α expression is markedly impaired *in vivo* [13,26]. It was therefore of importance to verify whether changes in the expression levels of the other splice variants could compensate for this defective regulation of p85 α PI 3-kinase during type 2 diabetes.

In this study, we describe the genomic map of the human p85 α gene (*grb-1*). Based on this organization, we have developed a quantitative reverse transcriptase competitive PCR (RT-cPCR) assay of the mRNA corresponding to the different splice forms. Expression of the regulatory subunits of PI 3-kinase was determined in skeletal muscle and adipose tissue biopsies of healthy subjects and type 2 diabetic patients taken before and after a 3 h hyperinsulinaemic clamp [13]. Furthermore, we have studied the mechanism by which insulin regulates the expression of the different mRNA variants using a model of human differentiated muscle cells in primary culture [27].

EXPERIMENTAL

Screening of databases

Online databases (EMBL, Genbank and DDBJ) were screened using the Blast program. Sequences were analysed further, in terms of contig organization, sequence alignments and translation of coding regions, using commercially available software.

Human skeletal muscle and adipose tissue samples

To investigate insulin action on gene expression, healthy lean subjects and type 2 diabetic patients were submitted to a 3 h euglycaemic hyperinsulinaemic clamp. Biopsies of vastus lateralis muscle and subcutaneous abdominal adipose tissue were taken

3 h before and at the end of the clamp, as described in detail previously [13]. Muscle samples were obtained, under local anaesthesia (2 % lidocain), by percutaneous biopsies using Weil Blakesley pliers. Abdominal subcutaneous adipose tissue was aspirated from the peri-umbilical area through a 15-gauge needle. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Total RNA were prepared as indicated previously [13].

Seven healthy lean volunteers (four women and three men; age, 41 ± 5 years; body mass index, 23 ± 1 kg/m²) and seven patients with type 2 diabetes mellitus (three women and four men; age, 51 ± 2 years; body mass index, 31 ± 2 kg/m²; haemoglobin a1c, 11.2 ± 0.5 %; duration of diabetes, 7 ± 1 years) participated in the present investigation. These subjects were involved in a study on the regulation by insulin of gene expression in skeletal muscle and adipose tissue that has been reported previously [13]. None of the control subjects had a familial or personal history of diabetes, obesity, dyslipidaemia or hypertension. All participants gave their written consent after being informed of the nature, purpose and possible risks of the study. The experimental protocol was approved by the Ethical Committees of the Hospices Civils de Lyon and performed according to French legislation.

During the hyperinsulinaemic clamp, insulin (Actrapid Novo, Copenhagen, Denmark) was infused at a rate of 450 pmol $\cdot$ m⁻² $\cdot$ min⁻¹, and euglycaemia was maintained by adapted infusion of 20 % glucose solution (Aguettant, Lyon, France) [13]. During the last hour of the clamp, insulinaemia plateaued at 935 ± 92 pM in the control group and at 1189 ± 56 pM in the type 2 diabetic patients. Under such conditions, the glucose-disposal rate was significantly lower in type 2 diabetic patients (4.0 ± 0.3 mg $\cdot$ kg⁻¹ $\cdot$ min⁻¹) than in control subjects (9.5 ± 0.7 mg $\cdot$ kg⁻¹ $\cdot$ min⁻¹, $P = 0.002$), indicating a marked state of insulin resistance in diabetic subjects [13].

Primary culture of human skeletal-muscle cells

Biopsies of the lumbar mass (erector spinae) muscle were taken during a surgical procedure with the consent of the subjects and the approval of the Ethical Committees of the Hospices Civils de Lyon. In the present study, biopsies were taken from three healthy subjects (two men and one woman; age, 43 ± 7 years; body mass index, 24 ± 1 kg/m²) with no familial or personal history of diabetes. The satellite cells were isolated from the muscle biopsy by trypsin digestion and were grown in Ham's F10 medium supplemented with 20 % fetal calf serum and 1 % antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin), as described previously in detail [27]. Confluent myoblasts were differentiated into myotubes in α -minimal essential medium containing 2 % fetal calf serum and 1 % antibiotics. Human myotubes were used 12–16 days after induction of the differentiation process. At this stage, most cells showed a multinucleated status that characterizes mature myotubes [28], and immunocytofluorescence studies demonstrated significant expression of myosin and of the striated-muscle-specific sarcomeric α -actin [27]. After overnight serum deprivation differentiated myotubes were incubated for 6 h with insulin and different inhibitors of the signalling pathway. Total RNAs were prepared using the RNeasy kit from Qiagen as previously indicated [29].

Quantification of the splice mRNA variants of *grb-1* gene

According to the determined genomic organization, six different mRNAs could be produced from *grb-1* (Figure 1). To quantify, by RT-cPCR, the mRNAs corresponding to the p85 α , p55 α and p50 α proteins, we designed and constructed a multi-specific

Figure 1 Genomic organization of the human *grb-1* gene

The locations of exons (white boxes) and introns (lines between exons) are indicated on an ≈ 80 kb region of chromosome 5q13. For clarity, the size of the intron 1 of p85 α (46436 bp between E1-85 and E2) has been reduced. Below the genomic map are represented the cDNAs encoding p55 α , p50 α and p85 α variants. The main protein domains are represented on the cDNAs by distinctly filled boxes. Untranslated regions are denoted by striped boxes. ATG and TGA represent initiation and termination codons, and the polyadenylation motif (AATAAA) is indicated at the 3'-ends of the cDNAs. Exons are numbered according to the previously reported mouse *pi3kr1* gene [14], except for the first specific exon of each variant (E1-85, E1-55 and E1-50 for the p85 α , p55 α and p50 α variant, respectively). Primers used for RT-PCR quantification are represented by white arrows and numbered according to Figure 2. The black arrowhead indicates the location of the eight-amino-acid insertion resulting from alternative splicing of exon E14 (*, and Figure 3). The vertical dotted line represents the limit of the common part of the different protein variants. BCR, breakpoint cluster region.

competitor DNA molecule. The organization of the competitor and the sequences of the primers are shown schematically in Figure 2. From muscle total RNA, a reverse-transcription reaction was performed using P1 as antisense primer, located in the common region (exon E9). A 345 bp PCR fragment was obtained using primers P1 and P3 (located in exon E1-50) and subcloned into the pGEM-T vector (Promega, Charbonnières, France). A 39 nucleotide-long fragment was deleted by restriction enzyme digestion (*XhoI/AvaII*). Then a 60 nucleotide-long fragment, corresponding to 30 nucleotides that are specific to the p55 variant (located in exon E1-55 and containing primer P4) and 30 nucleotides specific to the p85 variant (located in exons 5 and 6 and containing primer P5) were added at the 5' end by PCR overlap extension. The resulting multi-specific DNA competitor molecule was subcloned into the pGEM-T vector and verified by sequencing. The sizes of the PCR products generated with the competitor and with the natural target mRNAs are indicated in Figure 2. To validate the RT-cPCR assays, known amounts of p85 α , p55 α and p50 α cDNA (obtained by PCR and subcloned in pGEM) were quantified by cPCR using the multi-specific competitor. The resulting dose-response curves were all linear with slopes close to 1, indicating similar PCR efficiency for the amplification of the competitor and the three target cDNAs [30].

For the RT-cPCR assays, reverse transcription was performed from 0.2 μ g of total tissue RNA using the common antisense primer P1 and a thermostable reverse transcriptase enzyme (*Tth*; Promega) under conditions that warranted optimal synthesis of the first-strand cDNAs [30]. RT medium (5 μ l) was used for the specific cPCRs using one of the four sense primers (P2, P3, P4 or

P5). Sense primer P2, located in the common exon 7, was used for the global quantification of all the mRNAs encoded by *grb-1*. During the PCR, sense primers that were 5'-end-labelled with Cy-5 fluorescent dye were utilized and the PCR products were analysed with automated laser fluorescence DNA sequencer (ALFexpress; Pharmacia, Uppsala, Sweden). The initial concentration of target mRNA was determined at the competition equivalence point, as described previously [30].

Presentation of the results and statistical analysis

All data in text and figures are presented as means $\pm$ S.E.M. Statistical significance of the results was determined using the non-parametric Mann-Whitney test when comparing groups of subjects. A non-parametric Wilcoxon test for paired values was used when comparing mRNA levels before and after clamping in the same group of subjects. The threshold for significance was set at $P < 0.05$.

RESULTS

Genomic organization of the human *grb-1* gene

Databank screening was performed with Blast-2 software using the human p85 α mRNA sequence (Genbank accession number M61906) as a probe. Two human chromosome 5 genomic sequences (AC008485 and AC016564) were found to contain the whole cDNA sequence. Alignment of the genomic and the messenger sequences revealed that *grb-1* spans over 73 kb with 15

Primers	Sequences	PCR product size			Corresponding mRNA variants
P1	5'-GAGATTCATTCCGG TAGTGG-3'	cDNA		Competitor	
P2	5'-ACTACTGTAGCCAAC AACGG -3'	P1/P2	295	256	all forms
P3	5'-CCGTTGA AATG CATAA CCTGC-3'	P1/P3	345	306	p50α
P4	5'- ATTGTGGCA CAGAC TTGATG -3'	P1/P4	361	336	p55α
P5	5'- ATTCTCAG CAGC CAGCTCTG-3'	P1/P5	418	366	p85α
P6	5'-CCAG CTGAG AAAGACG AGAG-3'	Without insert		With insert	all forms
P7	5'-AAAGTG CCATCTCGCTTCCC-3'	P6/P7	224	248	

Figure 2 Competitor and primers used for RT-cPCR mRNA assays

Schematic map of the competitor cDNA (366 bp) cloned into the pGEM-T vector. It contains specific regions of the p50α, p55α and p85α mRNA variants at the 5'-end and 276 bp of common sequence. The locations of the primers used for the RT-cPCR are indicated (white arrows). The arrowhead indicates the 39 bp deletion (*XhoI/AvaII* digestion). The table shows the sequences of the primers and the sizes of the various PCR products. P6 and P7 were used to amplify a region containing exon E14 (see Figure 1) and sizes corresponding to the forms with and without the 24 bp insert are indicated.

Figure 3 Alternative splicing of exon E14

Schematic representation of the alternative splicing of the intron between exons E13 and E14. The two *tag* 3'-acceptor sites (in italic) are separated by 24 nucleotides (shaded box). The alternative protein sequences that can be generated are indicated. Asterisks indicated putative serine phosphorylation sites [15].

exons (Figure 1). All introns were flanked by splicing consensus sequences (5'-GT and AG-3') and their individual sizes ranged from 95 to 46436 bp (Figure 1). This organization was further confirmed by examination of the draft assembly of the human genome (the 7th October 2000 release) available within the Human Genome Browser (<http://genome.ucsc.edu>).

Comparison of the mRNA sequences of the p85α, p55α and p50α variants revealed that the common C-terminal part of the proteins is encoded by exons E7–E15. The p85α-specific part

(containing the SH3 domain) corresponds to a sequence encompassed by exons E1–E6. From the specific N-terminal protein sequences of p50α (six amino acids) and p55α (34 amino acids and the cDNA sequence reported by Antonetti et al. [15]), the sequences of the specific exons were found on the genomic map between exons E6 and E7 (Figure 1). Exons E1–55α and E1–50α both contain a splicing consensus at their 3'-ends. Their genomic location was further confirmed by screening human EST data-banks using p55α- and p50α-specific cDNA sequences as probes.

Figure 4 Relative mRNA expression of *grb-1* splice variants in human muscle and adipose tissue

The mRNA levels of the variants were quantified by RT-cPCR and expressed as a percentage of total *grb-1* transcripts in skeletal muscle (A) and abdominal subcutaneous adipose tissue (B). No significant differences were found between control (open boxes) and type 2 diabetic (closed boxes) subjects. Data are presented as means $\pm$ S.E.M., $n = 7$.

The coding sequence of the eight-amino-acid-long insert described by Antonetti et al. [15] was localized at the 5'-end of exon E14. This location is in agreement with the cDNA clones. Because the insert sequence is flanked by consensus splicing sites (TAG), the presence of the eight additional amino acids in the common C-terminal part of the proteins is most probably controlled by alternative splicing of the intron between exons E13 and E14 (Figure 3).

From the proposed genomic organization, it can thus be postulated that *grb-1* gene may encode at least six different proteins, namely p85α PI-3 kinase, p55α PI-3 kinase and p50α

PI-3 kinase containing or not the insert of eight amino acids in the inter-SH2 region.

Expression of the different mRNA variants in human tissues

Measurements of the expression levels of the different variants was performed by RT-cPCR on total RNA preparations from skeletal muscle and subcutaneous adipose tissues of healthy subjects and type 2 diabetic patients. We first looked at the relative abundance of the mRNA variants containing the eight-amino-acid insertion at the 5'-end of exon E14. Because the insertion occurs in the common part of the messengers, it could be present in p85α, p55α and p50α variants. After reverse transcription with antisense primer P7 (Figure 1), the relative amount of the form with the insert was estimated by PCR, using primers P6 and P7 (Figures 1 and 2). Both in muscle and in adipose tissue, amplification gave rise to a major PCR product of 224 bp, corresponding to the forms without the insert, while a tiny band was visible at 248 bp (results not shown). Relative quantification of the PCR products using Cy-5 fluorescent dye-labelled P6 primer and PAGE revealed that the insert-containing variants represented less than 5% of the *grb-1* messengers in human muscle and adipose tissue (results not shown). To further confirm this result, we tried to quantify the amounts of p85α, p55α and p50α mRNAs containing the insert, using an antisense primer located within the 24 bp insert during the reverse-transcription reaction, and in the competitor during cPCR. Using this strategy, the amount of amplified mRNA was under the limit of the RT-cPCR assay (10^{-20} mol), both in muscle and in adipose tissue. Moreover, no change in the relative expression of the forms with or without the insert was observed in tissue samples obtained after 3 h of hyperinsulinaemic clamp (results not shown). These results indicated therefore that the level of mRNA variants containing the insert in exon E14 was extremely low, if there were any, in the tested human tissues.

Under such conditions, quantification of the mRNA levels of p85α, p55α and p50α was performed by RT-cPCR using the competitor cDNA described in Figure 2 and the antisense primer P1 during the reverse-transcription step. Figure 4 shows the relative abundance of the three mRNA variants in skeletal muscle (Figure 4A) and subcutaneous adipose tissue (Figure 4B) in control and type 2 diabetic subjects. There was no difference in the absolute mRNA expression of any of the variants between control subjects and type 2 diabetic patients in either tissue. The three forms could be measured in skeletal muscle (Figure 4A). This tissue was characterized by high mRNA expression of p50α (45–55% of the total *grb-1*-derived mRNAs), followed by p85α (35–40%) and minor expression of p55α (8–12%). In adipose tissue (Figure 4B) the relative expression of the variants markedly differed from what was seen in muscle, with p85α representing about 80% and p50α about 20% of the mRNAs. The mRNA levels of p55α were below the detection limit of the RT-cPCR assay.

Regulation of *grb-1* mRNA variant expression by insulin *in vivo*

The absolute mRNA levels of the three variants was determined in skeletal-muscle biopsies obtained before and at the end of a 3 h hyperinsulinaemic clamp. Figure 5 shows that insulin significantly increased the mRNA expression of the three variants in the muscle of healthy control subjects. Insulin induced a $\approx$ 2-fold increase in the expression of p85α mRNA (1.6 ± 0.6 versus 3.0 ± 1.0 amol/ μ g of total RNA, before and after clamping respectively; $P = 0.028$), and a $\approx$ 2.5-fold increase in the expression of p50α (2.1 ± 0.6 versus 4.9 ± 1.0 amol/ μ g of total RNA; $P = 0.042$). The magnitude of the up-regulation of the

Figure 5 Regulation by insulin of *grb-1* splice-variant expression in human skeletal muscle

The mRNA levels of p85α, p55α and p50α variants were quantified by RT-cPCR in the skeletal muscle of seven control subjects (○) and seven type 2 diabetic patients (●), before and after a 3 h hyperinsulinaemic euglycaemic clamp, as indicated in the Experimental section. Statistical analyses were performed using the non-parametric Wilcoxon test for paired values. Data are expressed in amol/μg of total RNA.

p55α variant was more pronounced (≈ 8 -fold; 0.3 ± 0.1 versus 2.3 ± 0.6 amol/μg of total RNA; $P = 0.018$).

We have previously shown that the regulation by insulin of p85α mRNA expression is impaired in the skeletal muscle of type 2 diabetic patients [13,26]. Here, we confirmed these results using different primers and cDNA competitor in the RT-cPCR assay (Figure 5). Furthermore, our results showed that the regulation by insulin of p50α was also altered in type 2 diabetic muscle (2.3 ± 0.6 versus 3.8 ± 0.9 amol/μg of total RNA, before and after clamping respectively; $P = 0.31$). In contrast, the mRNA levels of p55α were still significantly increased during insulin infusion (0.6 ± 0.2 versus 1.9 ± 0.5 amol/μg of total RNA; $P = 0.046$). However, the magnitude of the effect of insulin (≈ 3 -fold) was reduced compared with the increase in p55α mRNA that was observed in control subjects (≈ 8 -fold).

Owing to the low yield of total RNA in adipose tissue (about 1 μg/100 mg of tissue [13]), quantification of mRNA variants before and after hyperinsulinaemic clamp was performed in five of the control subjects only. Insulin increased p85α mRNA (8 ± 2

Figure 6 Regulation by insulin of *grb-1* splice-variant expression in human muscle cells in primary culture

Myotubes obtained from control subjects were incubated for 6 h in the presence of the indicated agents. Ins, 10^{-7} M insulin; LY, 50 μM LY294002; Rapa, 10^{-8} M rapamycin. Data are expressed as means $\pm$ S.E.M. from six separate experiments. * indicates a significant difference compared with basal values ($P < 0.05$).

versus 36 ± 9 amol/μg of total RNA; $P = 0.043$), and p50α mRNA (1.6 ± 0.4 versus 4.4 ± 1.4 amol/μg of total RNA; $P = 0.043$) in adipose tissue. The expression of p55α mRNA remained undetectable after 3 h of insulin infusion (results not shown).

Regulation of *grb-1* mRNA variant expression by insulin in human primary muscle cells

We have previously shown that insulin enhances the transcription of the p85α variant through the PI 3-kinase/protein kinase B/p70 S6 kinase pathway in primary cultures of human skeletal muscle [27]. Figure 6 shows the expression of the three *grb-1* mRNA variants in differentiated myotubes from healthy subjects. About 80% of *grb-1* transcripts corresponded to the p85α

variant in this cell model, whereas the two other forms represented about 10% each. Insulin (10^{-7} M for 6 h) significantly increased the mRNA levels of the three variants (Figure 6). Inhibition of the PI 3-kinase activity with LY294002 (50 μ M) or indirect inhibition of p70 S6 kinase activity with rapamycin (10^{-8} M), which inhibits mammalian target of rapamycin (mTOR) activity, completely abolished the effect of insulin on the mRNA expression of the three forms (Figure 6). These results suggested that insulin stimulation of the three *grb-1* variants' expression may require the PI 3-kinase/p70 S6 kinase pathway.

DISCUSSION

From genomic databases and from the recently available human genome draft sequence, we defined the complete organization of the *grb-1* gene that encodes the p85 α regulatory subunit of PI 3-kinase. The human gene spans over 73 kb on chromosome 5q13 and its intron/exon organization is highly homologous to what was reported for the murine *pi3kr1* gene [14,16]. Analysis of the gene organization confirmed that *grb-1* can produce at least six mRNA variants by alternative splicing [14–16]. Exons E1–E5 encode the unique p85 α N-terminal region (residues 1–333) which contains an SH3 domain, a proline-rich domain and a domain homologous to the breakpoint cluster region (BCR) gene product [1]. Exons E7–E15 encode the common part of different protein isoforms (p85 α , p55 α and p50 α) which contains the two SH2 domains that interact with the phosphorylated tyrosine residues of upstream signalling molecules, and the p110 binding domain [1]. Moreover, alternative splicing of exon E14 could generate additional forms with a small insert of eight amino acid residues in the inter-SH2 region. This insert contains two serines in consensus phosphorylation sites [15]. Finally, exons E1–50 α and E1–55 α are located between E6 and E7 and encode the unique N-termini of p50 α (six amino acids) and p55 α (34 amino acids), respectively.

Because of the major role of PI 3-kinase in insulin action, the expression pattern of the different variants generated by *grb-1* or *pi3kr1* genes has been evaluated in skeletal muscle and adipose tissue of various animal models and in humans. However, the literature is rather confused. Using Western blotting, it was reported that p85 α was the major protein variant in mice skeletal muscle whereas p50 α was a minor form, and p55 α was not expressed [23]. In another study, however, the three variants were detected in the muscle of lean or obese (*ob/ob*) mice, with a slight predominance of the lower-molecular-mass species [31]. In rat skeletal muscle, conflicting results have been also reported. The p85 α protein variant was found to be the most abundant in the muscle of Wistar rats [16] and in the muscle of lean and fat Zucker rats [32]. Very low expression of p55 α was reported and p50 α was not detectable in these models [32]. In contrast, others showed that p55 α was about 10-fold more abundant than the p85 α protein in Sprague–Dawley rat muscles [15]. In human muscle, five different regulatory proteins derived from the *grb-1* gene have been detected [20]. Forms that migrated at 46 and 48 kDa seemed more abundant than the p85 α protein, and faint bands were detected at 53 and 54 kDa. This might suggest that p50 α is the more abundant form in human skeletal muscle and that p55 α is expressed at low levels. However, using specific antibodies, the p50 α protein was shown to correspond to the band that migrated at 46 kDa. The other proteins remained to be identified [20]. Regarding adipose tissue, less information was available, and it appears that p85 α is predominantly expressed in rat fat depots and in rodent adipose cell lines [15,19,33].

At the mRNA level, quantification of the relative expression of the different variants was complicated by the presence of several

bands using Northern blotting, and also to the low mRNA expression levels of the splice variants in skeletal muscle [15,34]. Here, we determined the expression profile of the different variants of *grb-1* gene in human skeletal muscle and adipose tissue, using validated RT-cPCR-based mRNA assay. This method allowed the precise quantification of each splice mRNA variant using specific PCR primers, after a single reverse-transcription reaction that was designed to generate first-strand cDNA from all the forms. In addition to the RT-cPCR assays, we determined the relative abundance of the forms containing the insert of 24 nucleotides (alternative splicing at the 5'-end of exon E14). The mRNA expression levels of these insert-containing variants were extremely low, when detectable, in muscle and adipose tissue. This strongly suggested that the regulatory subunits of PI 3-kinase with the additional eight amino acids are probably not produced in human muscle and adipose tissue *in vivo*. Regarding the expression levels of the three major mRNA variants, we found that p85 α and p50 α were the more abundant in skeletal muscle, whereas the p85 α variant was expressed predominantly in adipose tissue. The mRNA levels of the p55 α variant were low in both tissues, particularly in adipose tissue where it could not be quantified. Further analysis of the abundance of the different protein forms is however needed to verify whether these mRNA expression profiles are translated at the protein level in human tissues.

It is still unclear how the different protein variants of the *grb-1* gene participate in insulin's mechanism of action in target tissues [1]. All forms can bind phosphorylated tyrosine residues of the IRS and activate p110 catalytic subunits of PI 3-kinase [35]. In cell lines, it was shown that the degree of PI 3-kinase activation in response to insulin stimulation was higher for p50 α than for p85 α and p55 α [16,35]. In insulin-stimulated rat muscle, both p85 α and p55 α were found to be associated with p110 α PI 3-kinase, but p85 α was associated with IRS-1 to a much greater extent than p55 α [15]. In human muscle, insulin greatly increased the levels of the 48 kDa regulatory subunit in anti-phosphotyrosine immunoprecipitates with smaller increases in the levels of p85 α and no change in the association of p50 α [20]. Altogether, these data did not allow us to draw a definite picture of the specific role of the different variants. The fact that different results have been obtained depending on the experimental model could reflect specific regulations of the PI 3-kinase signalling system [1]. It should be noted also that the mechanisms by which the regulatory subunits are differentially recruited into PI 3-kinase complexes are not known. The unique N-terminal regions of the variants are likely to contribute, maybe through interactions with additional cellular proteins [35–38]. In addition, the expression of the variants, and therefore relative abundance of the different forms, could also be determinant in the regulation of the PI 3-kinase pathway. This hypothesis is supported by the tissue-specific expression pattern of the splice forms and also by the recent demonstrations that the relative expression of the different variants is modified in the livers of obese rodents [31,32].

One important new finding of our study is the demonstration that the basal steady-state mRNA expression of the different *grb-1* splice variants is not impaired in muscle or adipose tissue of type 2 diabetic patients. Using a different mRNA assay we previously reported that the expression of p85 α was not altered in skeletal muscle of type 2 diabetics [13,39]. These data were in agreement with protein determination by Western blotting showing that the amount of p85 α is not different in control and type 2 diabetic muscle [40]. Our present data provide evidence that there is no difference in the mRNA expression of the p50 α and p55 α variants. Therefore, since the mRNA levels of p110 α

and p110 β catalytic subunits of PI 3-kinase are also not altered [13], these results strongly suggest that there is no major defect in the basal expression of the different PI 3-kinase subunits involved in insulin action in skeletal muscle of type 2 diabetic patients. It can thus be speculated whether the differential recruitment of the subunits participates in the altered PI 3-kinase pathway observed in this pathology [5,6]. Also, changes in the expression of p85 β or p55 γ , which have also been suspected to participate in insulin signalling [1,21], cannot be excluded.

We have demonstrated previously that *grb-1* expression is regulated by insulin both *in vivo* [41] and *in vitro* [27] in human skeletal muscle. One of the goals of this study was to verify whether this regulation was restricted to p85 α or was shared by all splice variants. The present data clearly showed that insulin increases the mRNA levels of the three major *grb-1* variants in muscle of control subjects, both *in vivo* during a 3 h hyper-insulinaemic clamp and *in vitro* in differentiated myotubes. In addition, experiments with classic inhibitors of insulin signalling suggested that the PI 3-kinase/p70 S6 kinase pathway is probably involved in insulin action on *grb-1* expression. However, because the inhibitors that have been used could also affect other pathways, like the novel protein kinase C isoforms, definite conclusions regarding insulin's mechanism of action could not be drawn.

We have demonstrated previously that insulin enhances p85 α transcription [27], so it could thus be assumed that the increase in p50 α and p55 α mRNA levels also reflects transcriptional regulation of *grb-1*. However, the mechanism underlying the generation of the different mRNA variants is not known. It could imply a single promoter controlling the production of all forms through alternative splicing process or specific promoters controlling the expression of each form. The presence of large upstream introns flanking specific exons E1-55 and E1-50 (particularly for E1-50) is consistent with this latest hypothesis. The higher magnitude of insulin effect on p55 α *in vivo* compared with the effect on the other forms and the different tissue expression patterns of the variants are in agreement with specific regulatory mechanisms. Further studies are therefore required to characterize the upstream regions of the different unique first exons and to verify whether the splicing process, stabilization of mRNA or change in transcription rates are involved in the differential regulation of the *grb-1* variants.

The regulation by insulin of p85 α mRNA expression is blunted in the skeletal muscle of type 2 diabetic patients [13,26]. In mice, the absence of p85 α can be compensated for by up-regulation of the other variants [23]. It was therefore of importance to verify whether the regulation by insulin of p55 α and p50 α was disturbed in type 2 diabetic muscle with regard to the impaired regulation of p85 α expression. Our data demonstrated that the effect of insulin on the expression of p55 α and p50 α mRNA variants is markedly reduced in the skeletal muscle of diabetic patients. These data thus indicate impaired regulation of *grb-1* expression *in vivo* in response to insulin infusion in type 2 diabetes.

We and others have previously reported defective regulation by insulin of the expression of additional genes in diabetic muscle [13,42–44], suggesting that the regulation of a cluster of genes involved in insulin action and glucose metabolism may be affected in type 2 diabetes. It is of note that all the genes that belong to this putative cluster are actually up-regulated by insulin [13,42–44]. The molecular mechanisms by which insulin represses the transcription of target genes begins to be well deciphered [45–49]. In contrast, little is known regarding how insulin enhances gene expression. Interestingly, the signalling pathway required for the up-regulation of at least two of these genes (hexokinase II and *grb-1*) requires PI 3-kinase and p70 S6 kinase ([27,50] and this

study). Therefore, type 2 diabetes could be characterized by a defect in the transduction of the insulin signal through this pathway to transcription factors involved in the regulation of specific targets. Understanding this mechanism will be an important step to identifying the causes of the altered regulation of gene expression in type 2 diabetes.

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