Insulin regulation of a novel WD-40 repeat protein in adipocytes

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Abstract

A 400 bp PCR product generated with degenerate primers derived from the glucagon-like peptide-1 receptor was used to screen a rat skeletal muscle cDNA library. The predicted amino acid sequence of the 978 bp open reading frame has a predicted Mr of 35 804, an estimated isoelectric point (pl) of 5·31 and contains seven WD-40 repeats, which are common to G-protein beta subunits (Gβ). Although chemically and structurally similar to Gβ subunits, the predicted amino acid sequence, when compared with the previously cloned Gβ isoforms, was found to be only 31–41% similar and thus was named Gβ-like (GβL, ‘Gable’). Western blotting of whole-cell lysates and immunoprecipitates of membrane and cytosolic fractions of HEK 293 cells stably overexpressing a carboxy-terminal His-tagged GβL indicates that the protein is cytosolic and that it migrates at 42 kDa. A 4 kb transcript was detected in all tissues surveyed by northern blotting; however, an additional 2 kb transcript was detected in testis. Expression of GβL mRNA was highest in the brain and testis, followed by lung, heart, kidney, skeletal muscle, spleen and liver. In addition, reverse transcriptase/PCR showed that several other tissues and cell lines express GβL. The ubiquitous nature of the tissue expression pattern of GβL is similar to that of the insulin receptor, which suggests that insulin may influence GβL expression. Indeed, GβL protein and mRNA levels, in fully differentiated 3T3-L1 adipocytes, were upregulated by insulin in a concentration-dependent fashion. These changes were highly sensitive to insulin stimulation, being minimally affected by doses as low as 0·1 nM and maximally elevated by 1 nM doses. These data suggest that insulin regulates GβL production and imply that some of the actions of insulin may be mediated, in part, by this novel intracellular protein.


Introduction

Members of the WD-40 family of eukaryotic proteins help to regulate intracellular signaling, RNA processing and degradation, gene expression, vesicular traffic and fusion, cytoskeletal assembly, and the cell cycle (van der Voorn & Ploegh 1992, Neer et al. 1994, Garcia et al. 1996). The earliest identified and most well-known members are the beta subunits of heterotrimeric guanine nucleotide-binding proteins (Gβ subunits of G-proteins). To date, five mammalian genes that encode Gβ isoforms have been identified. The corresponding proteins are highly homologous and all contain seven equally well-conserved WD-40 motifs (Hamm & Gilchrist 1996).

All proteins within the WD-40 family contain repeating sequences that are separated by approximately 40 amino acids. Each WD-40 repeat consists of two signature sites: a poorly conserved site A that usually contains a tryptophan and histidine (GH) pair and a relatively well-conserved site B that usually contains a glycine and histidine (GH) pair. Although every WD-40 family member contains four to eight of these repeating motifs, the separating distance and the actual sequence of individual repeats are highly variable. Most of these sites are separated by 36–46 amino acids; however, they are often separated by over 50. The functional diversity of WD-40 proteins suggests that the motif itself does not necessarily confer any particular function; rather, it contributes to the formation of anti-parallel β-strands that stabilize three-dimensional loops or β-propellers (van der Voorn & Ploegh 1992, Neer et al. 1994, Garcia et al. 1996). Although functionally distinct, many of these structural cognates form multiprotein complexes via non-covalent linkages between their β-propellers and other proteins including intracellular effectors, enzymes or even transmembrane receptors.

In an attempt to identify new G-protein coupled receptors (GPCRs) in muscle, rat heart cDNA was analyzed by a PCR with degenerate primers against the glucagon-like peptide-1 receptor. One of the resulting PCR products contained several regions that were signifi-
cantly homologous to Gβ subunits and to other proteins within the WD-40 superfamily. Reported herein is the isolation and characterization of a novel cDNA clone named GβL (‘Gable’), for Gβ-like, whose protein and mRNA levels are upregulated by insulin.

Materials and Methods

Isolation of cDNA clones encoding GβL

Total RNA from cardiac and skeletal muscle was extracted using guanidine thiocyanate and CsCl-centrifugation (Chirgwin et al. 1979). Reverse transcriptase/PCR (RT-PCR) was performed with Moloney murine leukemia virus reverse transcriptase and oligo-dT primers (both from GibcoBRL/Life Technology, Gaithersburg, MD, USA) in the first reaction and in the second with recombinant Amplitaq DNA polymerase (Perkin-Elmer, USA) in the absence or presence of a fivefold excess of competing peptide in RIPA buffer (50 mM Heps (pH 7-4), 267·6 mM NaCl, 2% Nonidet P-40, 0·2% Na dodecyl sulfate (SDS), 1% Na-deoxycholate, 2 mM Na-orthovanadate, 200 mM NaF, 1 mM benzamidine, 1 mM Pefabloc, 2 µg/ml leupeptin and 8 µg/ml aprotinin) containing protein A/G agarose that was preblocked in 0·01% SDS and transferred onto 0·2 µm polyvinylidene difluoride (PVDF) membranes (all from Novex, Gaithersburg, MS, USA). The membranes were blocked in 5% non-fat milk prepared in 20 mM Tris.HCl (pH 7·5), 137 mM NaCl and 0·1% Tween-20 (TBST), probed with affinity-purified GβL antiserum (1:2000) in 1% non-fat milk and then probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antiserum (HRP-GAR, 1·5000; Amersham, Piscataway, NJ, USA) in TBST. Positive immunoreactive reactions were visualized with Enhanced Chemiluminescence (ECL) detection reagents (Amersham).

Characterization of GβL antisera

Anti (α)-GβL was generated against a dodecapeptide (KSSNPGESSRGW) that is located within the carboxy-terminal region of GβL and is not contained within any other known proteins, as determined by a BLAST search. The antigenic peptide was cross-linked to BSA (BSA-pep) with bis (2-(succinimidooxy-carbonyloxy)-ethyl) sulfone (Pierce, Rockford, IL, USA). BSA-pep was then subsequently cross-linked to epoxy aminohexate sepharose with N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide HCl and used to affinity-purify GβL. BSA and BSA-pep (75 and 7·5 pmol) were then immunoprecipitated in the absence or presence of a fivefold excess of competing peptide in RIPA buffer (50 mM Heps (pH 7-4), 267·6 mM NaCl, 2% Nonidet P-40, 0·2% Na dodecyl sulfate (SDS), 1% Na-deoxycholate, 2 mM Na-orthovanadate, 200 mM NaF, 1 mM benzamidine, 1 mM Pefabloc, 2 µg/ml leupeptin and 8 µg/ml aprotinin) containing protein A/G agarose that was preblocked in 0·1% BSA. Immunoprecipitated proteins were separated by means of reducing SDS-PAGE on 4-12% gradient gels and transferred onto 0·2 µm polyvinylidene difluoride (PVDF) membranes (all from Novex, Gaithersburg, MS, USA). The membranes were blocked in 5% non-fat milk prepared in 20 mM Tris.HCl (pH 7·5), 137 mM NaCl and 0·1% Tween-20 (TBST), probed with affinity-purified GβL (1·2000) in 1% non-fat milk and then probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antiserum (HRP-GAR, 1·5000; Amersham, Piscataway, NJ, USA) in TBST. Positive immunoreactive reactions were visualized with Enhanced Chemiluminescence (ECL) detection reagents (Amersham).

Transfection of HEK 293 cells

Full-length rat GβL cDNA was generated by RT-PCR amplification of 5 µg rat brain RNA with gene-specific primers (forward, 5’-CTA AGG CAG AGT GCA GAG CG-3’; reverse, 5’-GCC CAC CAC GCT GTC GTT G-3’). After an initial denaturation at 94 °C for 4 min, rat brain cDNA was amplified by PCR for 30 cycles of the following regime: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. GβL cDNA was then gel-purified and subcloned into the pcDNA3-1-V5/His/TOPO TA cloning vector (pcDNA31-GβL/V5/His). HEK 293 cells were plated in Dulbecco’s Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum.
(FBS). Once they were 70% confluent in 25 mm² flasks, cells were preincubated for 1 h in serum-free DMEM and transfected with 2.7 µg pcDNA3-GßL/V5/His and Lipofectamine Plus reagent (GibcoBRL/Life Technology). The transfection was terminated after 3 h by replacing the serum-free DMEM with DMEM/10% FBS. A polyclonal stable cell line was derived by the addition of 500 µg/ml G418 to the culture medium 48 h after transfection and by subsequently culturing these cells in the presence of G418 for approximately 3 weeks.

Western blotting and immunoprecipitation

Whole-cell lysates from wild-type and transfected cells were solubilized in 1·5 × reducing SDS-PAGE sample buffer and analyzed by Western blotting as described. To determine the cellular localization of GßL, transfected cells were washed three times in PBS and quickly frozen in liquid nitrogen, scraped and solubilized in 1·5 × reducing sample buffer and then analyzed by western blotting with antisera against GßL (common (c) Gß or Gßq/11 (Santa Cruz)) as described above.

Changes in GßL mRNA levels were determined by semi-quantitative RT-PCR. RNA (5 µg) from each sample was reversed-transcribed as described above. Before amplification of sample cDNA, the linear portion of the amplification curves for actin and GßL transcripts were first defined by cycle titration PCR on control-cell cDNA with GSPs (actin forward, 5’-TAT GGA GAA GAT TTG GCA CC-3’; actin reverse, 5’-TCA TCG TAC TCC TGC TTG C-3’; GßL = pHB1 primers as above). Once the optimal cycle number had been identified, 10 µl cDNA from each sample was diluted into aliquots of a PCR master-mix buffer containing 50 µM dNTP, 2·5 U Taq DNA polymerase and 0·04 µCi [32P]deoxycytosine triphosphate/µl (Amersham). Samples were then divided in half before the addition of actin- or GßL-specific primers (to a final concentration of 0·2 µM) and were then amplified separately (actin, 25 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; GßL, 32 cycles). Amplicons were resolved on 6% polyacrylamide Tris–borate EDTA gels, which were subsequently dried and exposed to film.

Statistical analysis

Differences between the means were determined by ANOVA coupled to Fisher’s Least Significance Difference for multiple mean comparisons. GßL transcript levels were normalized to those of actin, and each insulin dose was repeated four separate times. Interassay variation was controlled by expressing ‘treated’ values as percentages of the controls.

Results

Isolation and analysis of GßL cDNA clones

A 400 bp PCR product was isolated using degenerate primers derived from the GLP-1 receptor. From this product, an internal 256 bp subsequence was used to isolate several partial clones of approximately 1·4–1·6 kb from a rat skeletal muscle cDNA library. Three of the largest clones were sequenced and each contained a large, but incomplete, open reading frame, the putative 3’-UTR with the polyadenylation signal sequence and 18 nucleotides of the poly(A) tail (Fig. 1). An additional 370 bp was obtained by 5’ RACE, and the remaining portion of the 5’-UTR (Fig. 1, italics), which could not be amplified by RT-PCR, was obtained by genome walking. The accession number of this sequence has been deposited in the GenBank database (accession no. AF051155).
The GβL open reading frame of 978 bp codes for a protein of 326 amino acids and contains a sufficient Kozak sequence surrounding the initiator codon (Kozak 1996). The predicted amino acid sequence of the GβL protein has a calculated Mr of 35 804 and an estimated isoelectric point (pI) of 5.31, properties that are similar to those of previously identified Gβ isoforms (Hamm & Gilchrist 1996). It contains seven WD-40 motifs that are separated from each other by 42–50 amino acids (Fig. 1).

Figure 1 Nucleotide and predicted amino acid sequence of GβL cDNA. Nucleotide numbering is relative to the adenosine in the initiator codon. Amino acid numbering (bold) is relative to the first methionine. The 5'-UTR sequence isolated from genomic DNA is shown in italics, residues fulfilling the site B portion of the WD-40 repeats are shaded, and in-frame stop codons and the polyadenylation signal sequence are in bold. The cDNA probe sequence used for Northern blotting and library screening and the immunogenic epitope used in generating anti-GβL are shown underlined.
these homologies are limited to the WD-40 site B motifs ($\Phi\Phi\Phi(D/N/K)\Phi\Phi\Phi(W/F/Y)(D/N/E/R/K)\delta$ where $\Phi=$ hydrophobic & A,T,G,S; $\delta=$ any; $\delta=$ non-charged) which are relatively well conserved (residues 15, 16) (Fig. 1, shaded). Potential 'GH' motifs of WD-40 site A ($\Phi\Phi\Phi(G/\Phi)(H/K)\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Φ
fully differentiated 3T3-L1 adipocytes that were stimulated with varying concentrations of insulin. Although the Gαq/11 and cGβL levels were unaffected by insulin, GβL protein levels were elevated in a concentration-dependent manner (Fig. 4A; representative blots of two separate experiments are shown). 3T3-L1 adipocytes were sensitive to doses of insulin as low as 0.1 nM and were maximally stimulated at 1 nM. In order to compare GβL mRNA values between treatment groups by semi-quantitative RT–PCR, the linear portion of the amplification curve for both GβL and actin transcripts was first identified (Fig. 4B). Samples were then amplified for a particular number of cycles that would allow for changes within the linear range (actin=25, GβL=32) and GβL transcript values were then normalized to those of actin. Insulin raised the GβL transcript levels in a concentration-dependent manner and the effect was maximal at concentrations at or above 1 nM insulin (Fig. 4C). Therefore, 3T3-L1 adipocytes appeared to be sensitive to concentrations of insulin of less than 1 nM. Changes in GβL mRNA levels were similar to the changes in protein levels; thus, the ability of insulin to upregulate GβL production is not due to an increase in translational efficiency per se, but appears to be due either to increased GβL gene transcription or to increased transcript stability.

Discussion

GβL possesses many structural features that are characteristic of Gβ subunits, including a similar molecular weight and isoelectric point as well as the presence of seven WD-40 repeating motifs. However, the low overall homology between GβL and any of the known Gβ proteins suggests that GβL, unlike many of the mammalian Gβ subunits, did not originate from the same ancestral gene and is therefore not a legitimate Gβ homologue. However, BLAST analysis did identify a yeast ribonucleoprotein, POP3, whose amino acid sequence is 60–4% similar and 44% identical to rat GβL. POP3 is a protein component of yeast RNase mitochondrial RNA processing and RNase P. These RNA–protein complexes are responsible for the post-transcriptional modification of primary rRNA and tRNA transcripts respectively. Although the level of homology shared by these two proteins does not necessarily suggest that they are true orthologues, it is indicative of functional conservation and suggests that GβL may either interact directly with RNA or even function as (or in conjunction with) an endoribonuclease.

Insulin is capable of altering the expression of many genes, both at a transcriptional and post-transcriptional level. Specifically, insulin stimulation increases the stability of farnesyltransferase α- and β-subunit message, which ultimately facilitates the farnesylation and membrane incorporation of p21 Ras (Goalstone & Draznin 1998, 1999). The metabolic actions of insulin are mediated, in part, by similar increases in glyceral 3-phosphate dehydrogenase and glucokinase mRNA stability (Iynedjian et al. 1989, Bhandari et al. 1991). Of particular interest is the ability of insulin to inhibit gluconeogenesis by inducing phosphoenolpyruvate carboxykinase (PEPCK) mRNA degradation (Christ et al. 1988, 1990, Heise et al. 1998). This
transcript-specific increase in RNase activity is prevented by actinomycin D, indicating that gene transcription is required. Since the structural similarities between GβL and POP3 suggest that the two proteins may possess similar actions, it is conceivable that insulin-induced changes in the stability of some transcripts, in particular PEPCK, may be influenced by GβL.

The ability of insulin to upregulate GβL protein and mRNA levels in 3T3-L1 adipocytes suggests that insulin is a potent regulator of GβL production and imply that GβL may contribute to the intracellular events associated with insulin receptor (IR) activation. Insulin-stimulated glucose transport, glycogen synthesis and cell growth are believed to occur primarily in response to the ligand-activated autophosphorylation of the IR beta subunits and the consequential phosphorylation and activation of cytosolic kinases and adapter proteins (Cheatham & Kahn 1995). However, earlier studies on the mechanisms of IR signaling indicated that some actions of insulin were mediated, in part, by pertussis-toxin-sensitive G-proteins (Heyworth et al. 1986, Gawler et al. 1987, Pyne et al. 1989, Butler et al. 1996, Krieger-Brauer et al. 1997). Although G-proteins traditionally associate with seven-transmembrane domain receptors (Bourne 1997), more recent studies have demonstrated both structural and functional links between G-proteins and the IR. (Jo et al. 1992, 1993a, b, Moxham & Malbon 1996, Krieger-Brauer et al. 1997, Zheng et al. 1998, Imamura et al. 1999). Therefore, some of the actions of insulin may be mediated by unconventional signaling events independent of the known IR signaling cascade. Although it remains to be determined whether GβL directly interacts with any of these known intermediates, the ability of insulin to raise GβL production implies that it is nevertheless important to the actions of insulin.

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