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 M_r of 35 804, an estimated isoelectric point (pI) of 5.31 and contains seven WD-40 repeats, which are common to G-protein beta subunits (G β). Although chemically and structurally similar to G β subunits, the predicted amino acid sequence, when compared with the previously cloned G β isoforms, was found to be only 31–41% similar and thus was named G β -like (G β L, '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 G β L 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 G β L 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 G β L. The ubiquitous nature of the tissue expression pattern of G β L is similar to that of the insulin receptor, which suggests that insulin may influence G β L expression. Indeed, G β L 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 G β L production and imply that some of the actions of insulin may be mediated, in part, by this novel intracellular protein.

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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 (G β subunits of G-proteins). To date, five mammalian genes that encode G β 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 glycine and histidine (GH) pair and a relatively well-conserved site B that usually contains a tryptophan and aspartate (WD)

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, Gaithersburg, MD, USA) and degenerate primers against the extracellular domain of the glucagon-like peptide-1 (GLP-1) receptor (forward, 5'-GTCGAATTG (EcoRI) GAA/G TAT/C A/CGI CAT/C CAA/G TGT/C C-3'; reverse, 5'-GCTCTAGA (XbaI) CCA/G TCC CAT/C TGA/G TGT/C TGT/C TG-3'). The amplification products were subcloned into pBluescript KS to obtain pHB1 following digestion with EcoRI and XbaI (GibcoBRL/Life Technology) and were eventually used to screen a Uni-Zap XR skeletal muscle cDNA library (Stratagene, La Jolla, CA, USA). Several partial clones of approximately 1.6 kb were isolated and each was found to contain the 3'-untranslated region (UTR) and a portion of the polyadenylated tail. Part of the remaining upstream sequence was determined with the 5' RACE (rapid amplification of cDNA ends) system (GibcoBRL/Life Technology) using the following gene-specific primers (GSPs): GSP1, 5'-ccg gca cta ttg act gcc gc-3'; GSP2, 5'-GCG CAC TGT GTG GTC ATA GCC TGC-3'; GSP3, 5'-CGGGATCC (BamHI) CGG TGG TAT TCA TTG TTT GAT GGC-3'. An additional 299 bp of the 5'-UTR were identified by genome walking (Clontech, Palo Alto, CA, USA) with GSP2 and GSP4 (5'-CGGGATCC (BamHI) CGC TCT GCA CTC TGC CTT AGT C-3'). All PCR products were subcloned into pBluescript KS following digestion with BamHI and SalI.

Tissue distribution of GβL expression

A multiple-tissue northern blot (Clontech) containing mRNA from rat heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis was probed with the radiolabeled pHB1 insert following EcoRI/XbaI digestion. Band intensities were quantified by electronic autoradiography (Packard Instruments, Meriden, CT, USA). GβL expression was also identified by non-quantitative RT-PCR of total RNA from rat epididymal, abdominal and brown fat,

small intestine, and pancreas, as well as from 3T3-L1 fibroblasts and adipocytes, L6-myotubes, Chinese hamster ovary (CHO) cells and rat insulinoma cells, as described above with primers internal to pHB1 (forward, 5'-CTG CAC GGC TCG CAT CTG GG-3'; reverse, 5'-CCG GCA CTA TTG ACT GCC GC-3').

Characterization of GβL antiserum

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 αGβL. 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 Hepes (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 benzamide, 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 αGβL (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 immunogenic 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 CAG 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 μ g pcDNA31-G β L/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 μ g/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 $\times$ reducing SDS-PAGE sample buffer and analyzed by Western blotting as described. To determine the cellular localization of G β L, transfected cells were washed three times in PBS and quickly frozen in liquid nitrogen. Cells were then scraped in a cell suspension buffer containing 20 mM Hepes (pH 7.6), 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM benzamidine, 1 mM Pefabloc, 2 μ g/ml leupeptin and 8 μ g/ml aprotinin and sheared through a 26-gauge needle. Lysates were then incubated on ice for 30 min with 20 μ g/ml DNase I before centrifugation at 20 000 $\times g$ for 30 min at 4 °C. The supernatant (cytosolic fraction) was then removed and CHAPSO was added to a final volume of 0.5%; the pelleted membrane fraction was then solubilized in the cell suspension buffer supplemented with 0.5% CHAPSO to a volume equal to that of the supernatant (1 ml) and centrifuged for an additional 15 min. Non-specific proteins from both crude fractions were first precleared with G β L preimmune serum (1:500) for 1 h at room temperature. Each fraction was then immunoprecipitated with α G β L (1:500), also for 1 h at room temperature. Whole-cell lysates and immunoprecipitated proteins were separated on 4–12% SDS-polyacrylamide gels and analyzed by western blotting with either HRP-conjugated anti(α)V5 or with α G β L as described above. The membrane harboring whole-cell lysates alone was first blotted with α V5-HRP, stripped (Chemicon, Temecula, CA, USA) and reblotted with α G β L. To confirm sufficient stripping of α V5-HRP, ECL reagents were applied to the membrane before reprobing with α G β L.

Insulin regulation of G β L production in 3T3-L1 adipocytes

3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% calf serum until 2 days post-confluency. Differentiation was induced by the addition of 0.5 mM isobutylmethylxanthine, 1 mM dexamethasone and 167 nM insulin to DMEM that was supplemented with 10% FBS. Cells were then incubated for 2 days in this differentiation medium, for 2 days in DMEM/10% FBS with insulin and for 2 days in DMEM/10% FBS. Fully differentiated cells were then preincubated for 48 h in

serum-free DMEM before the addition of 0, 0.01, 0.1, 1, 10 and 100 nM insulin. After incubation for 16 h, cells were washed three times in PBS, rapidly frozen in liquid nitrogen, scraped and solubilized in 1.5 $\times$ reducing sample buffer and then analyzed by western blotting with antisera against G β L (common (c) G β or G α q/11 (Santa Cruz)) as described above.

Changes in G β L mRNA levels were determined by semi-quantitative RT-PCR. RNA (5 μ g) from each sample was reversed-transcribed as described above. Before amplification of sample cDNA, the linear portion of the amplification curves for actin and G β L 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'; G β L=pHB1 primers as above). Once the optimal cycle number had been identified, 10 μ l cDNA from each sample was diluted into aliquots of a PCR master-mix buffer containing 50 μ M dNTP, 2.5 U *Taq* and 0.04 μ Ci [³²P]deoxycytosine triphosphate/ μ l (Amersham). Samples were then divided in half before the addition of actin- or G β L-specific primers (to a final concentration of 0.2 μ M) 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; G β L, 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. G β L 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 G β L 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).

-360																				ATTCTAGCTT	-351
-350	GGTTGGTAGG	GTAGAGGATT	GTGTATGTCG	GTGTGGGGTG	AAGGGCAACG	ACAGTGTAA	CTTTTCTTGG														-281
-280	AGCTAAGCCT	GGAAAA TAGA	CAGCAGCAC	GTGTTT GTAG	TTCCCA TGAG	TTCAAGGCT G	AGGTGCGGCG														-211
-210	TGACAGCCAC	TCCGCGCACG	ATCACTTCTG	GAGTTCTTCA	TCTCAGAACT	CTCTTATCCT	TGTCACATCC														-141
-140	CCCACCTCCT	GGACCCATGG	TCTCGCCCCG	AGTTACTATC	GATAACTCTA	CGCTCGGGCC	GGCTTCTATC														-71
-70	GACTCGCTCC	TGCTCGCTCC	GCCGCTCTGG	ACTAAGGCAG	AGTGCAGAGC	GTGTGTTAAG	CCATCAAACA														-1
1	M	N	T	T	P	G	T	V	G	S	D	P	V	I	L	A	T	A	G	Y	20
1	ATG	AAT	ACC	ACC	CCA	GGC	ACA	GTG	GGC	AGT	GAC	CCT	GTC	ATC	TTA	GCA	ACT	GCA	GGC	TAT	60
21	D	H	T	V	R	F	W	Q	A	H	S	G	I	C	T	R	T	V	Q	H	40
61	GAC	CAC	ACA	GTG	CGC	TTT	TGG	CAG	GCT	CAC	AGT	GGA	ATC	TGC	ACT	CGA	ACA	GTG	CAG	CAT	120
41	Q	D	S	Q	V	N	A	L	E	I	T	P	D	R	S	M	I	A	A	T	60
121	CAG	GAC	TCT	CAG	GTG	AAT	GCA	TTG	GAG	ATC	ACT	CCA	GAC	CGG	AGC	ATG	ATT	GCT	GCT	ACA	180
61	G	Y	Q	H	I	R	M	Y	D	L	N	S	N	N	P	N	P	I	I	S	80
181	GGT	TAC	CAA	CAT	ATC	CGC	ATG	TAT	GAT	CTC	AAC	TCC	AAT	AAC	CCC	AAC	CCC	ATC	ATC	AGT	140
81	Y	D	G	V	S	K	N	I	A	S	V	G	F	H	E	D	G	R	W	M	100
141	TAT	GAC	GGA	GTC	AGT	AAG	AAC	ATC	GCA	TCA	GTG	GGT	TTT	CAT	GAG	GAT	GGC	CGC	TGG	ATG	300
101	Y	T	G	G	E	D	C	T	A	R	I	W	D	L	R	S	R	N	L	Q	120
301	TAT	ACA	GGT	GGA	GAG	GAC	TGC	ACG	GCT	CGC	ATC	TGG	GAC	CTC	AGG	TCC	CGG	AAC	CTG	CAG	360
121	C	Q	R	I	F	Q	V	N	A	P	I	N	C	V	C	L	H	P	N	Q	140
361	TGT	CAG	CGT	ATC	TTT	CAG	GTG	AAT	GCA	CCC	ATT	AAT	TGC	GTG	TGT	CTA	CAT	CCC	AAC	CAG	420
141	A	E	L	I	V	G	D	Q	S	G	T	S	H	I	W	D	L	K	T	D	160
421	GCA	GAA	CTC	ATC	GTG	GGT	GAC	CAG	AGC	GGC	ACG	AGC	CAC	ATC	TGG	GAC	CTG	AAG	ACA	GAC	480
161	H	N	E	Q	L	I	P	E	P	E	F	S	I	T	S	A	H	I	D	P	180
481	CAC	AAT	GAG	CAG	CTG	ATC	CCT	GAG	CCT	GAG	TTT	TCC	ATC	ACA	TCT	GCC	CAC	ATT	GAC	CCC	540
181	D	A	S	Y	M	A	A	V	N	S	A	G	N	C	F	V	W	N	L	T	200
541	GAT	GCC	AGC	TAC	ATG	GCG	GCA	GTC	AAT	AGT	GCC	GGA	AAC	TGC	TTT	GTC	TGG	AAC	CTG	ACG	600
201	G	G	I	G	D	E	V	T	Q	L	I	P	K	T	K	I	P	A	H	T	220
601	GGG	GGC	ATT	GGT	GAC	GAG	GTG	ACT	CAG	CTC	ATC	CCC	AAG	ACC	AAG	ATC	CCA	GCC	CAC	ACG	660
221	R	Y	A	L	Q	C	R	F	S	P	D	S	T	L	L	A	T	C	S	A	240
661	CGC	TAC	GCC	CTG	CAG	TGC	CGC	TTC	AGC	CCT	GAC	TCC	ACG	TTA	CTT	GCG	ACC	TGT	TCG	GCT	720
241	D	Q	T	C	K	I	W	R	T	S	N	F	S	L	M	T	E	L	S	I	260
721	GAC	CAG	ACG	TGT	AAA	ATC	TGG	AGG	ACA	TCC	AAC	TTC	TCC	CTG	ATG	ACA	GAA	CTC	AGC	ATC	780
261	K	S	S	N	P	G	E	S	S	R	G	W	M	W	G	C	A	F	S	G	280
781	AAG	AGT	AGT	AAC	CCC	GGA	GAG	TCA	TCC	CGT	GGC	TGG	ATG	TGG	GGC	TGC	GCC	TTC	TCA	GGG	840
281	D	S	Q	Y	I	V	T	A	S	S	D	N	L	A	R	L	W	C	V	E	300
841	GAT	TCC	CAG	TAT	ATC	GTC	ACA	GCT	TCT	TCA	GAC	AAC	CTG	GCC	CGG	CTC	TGG	TGT	GTA	GAG	900
301	T	G	E	I	K	R	E	Y	G	G	H	Q	K	A	V	V	C	L	A	F	320
901	ACT	GGA	GAG	ATC	AAG	AGA	GAG	TAT	GGT	GGC	CAT	CAG	AAA	GCT	GTC	GTC	TGC	TTG	GCC	TTC	960
321	N	D	S	V	L	G															326
961	AAC	GAC	AGC	GTG	CTG	GGC	TAG														1120
1121	CCTGGTCTAG	TGGCTCTAAG	AGGATGTACC	TGAACAATAC	CCTGCTCCAA	GAGAGACTCA	GGTCAGCCTC	1190													
1191	TGCATATCAG	GCCAGTGGGA	CCTGACCCGA	CTACCCCTCGG	CCCCTCTTGG	CCAACCTAGA	CTTTTGTGCT	1260													
1261	ACTCTACCCT	GGGACTTTCT	CCGTGCCCAG	ATCTGTTGTC	AGAAAGCTCA	CAAAGCCAGG	CTGCATGTGC	1330													
1331	CTGGATTGGG	AGTCTTCTCC	CTTTAGAGAG	ACGACACCTA	GAGTGTGTGG	AACCTGTTGAG	GACATCGGAG	1400													
1401	GTTGATGCCT	GTATCCGAGA	GCTGACAGGT	ACTGGCCCTC	CTGCCCCTCT	TCCACTCTGC	ATACCCCTGGA	1470													
1471	AGAGGTGCTT	ACCTTGGGAG	GGCTGTATGT	GAAACCAAGA	AGCCCTCCAG	CCACGGCCCT	TGCAGTGAGC	1540													
1541	AGTGTGAGCA	TCAGCAGCAA	GCCTGCTGCT	CTCCAGCCCC	TGTAGCAGGG	TTTGGTCTTC	ACCTGAGGGC	1610													
1611	ACAGTACTAG	CTGTGGGGCT	TCGGGCTTAG	AGACTCGGGA	GCCGACTTGC	TCAGGGAAAG	GGCTGAGGAT	1680													
1681	GGTAGGTGGT	GACTCCTGTC	TCCTCCTATG	GCATCCCTGA	GTTCTTGTCT	TGGGGGTGGT	AGACACTTAC	1750													
1751	CTCAACAGGC	TTGGACCTGA	GGCTCTCAGT	GAGGATGTGC	CCCTGCTTCA	TGCTGCAGCC	CCTGCTGACA	1820													
1821	ATGCACCTGAT	TGTTGCTGAG	CCCTTGGGAG	AGTCAGCCAG	GGAAACCTCA	GGATGCCTTT	GATGACTCGA	1890													
1891	ACTGTATCCT	CCCCCTCCAA	CCCCACCGCT	TCCTTCCCA	CCAGTATCTG	ATAGCCCTGC	TCAGAGCCAG	1960													
1961	GACAACCCCT	TCCCCCACTT	GTGATTGCTG	GTTACCAAGT	TTGTCCCTGG	ACTCTTGGCC	TGTGCAGAAC	2020													
2021	TAGGATTTT	GGAGTGTGTG	TGAGCTGGCC	TTGATGTTAG	AATTGGCTAG	GTCTTGGCCA	GGGTGACTTT	2090													
2091	TGCTGTGGTG	GGCAGATTTT	TTTCTTGGTG	TGTTACCTCT	GAGGCTCTAG	CTAGACACAG	GAACCATCAA	2160													
2161	TAAAGGGGTA	TGAACCATCA	G					2181													

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 αGβL are shown underlined.

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 M_r 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). BLAST analysis of the GβL nucleotide or amino acid sequence indicates that it shares significant homology with many WD-40-containing proteins, including the five mammalian Gβ subunits. However, GβL is only 24–32% identical and 31–41% similar to Gβ1–5 overall and most of

these homologies are limited to the WD-40 site B motifs (5 Φ _x(D/N/K)_{xx} Φ _x Φ (W/F/Y)(D/N/E/R/K) δ where Φ =hydrophobic & A,T,G,S; x=any; δ =non-charged) which are relatively well conserved (residues 15, 16) (Fig. 1, shaded). Potential 'GH' motifs of WD-40 site A (Φ _x(G/ Φ)(H/K)_{xxx} Φ _{xx} Φ) were also identified upstream of the last six site B motifs. These are AH (residues 29, 30), SK (residues 85, 86), LH (residues 136, 137), AH (residues 176, 177), AH (residues 218, 219) and IK (residues 260, 261).

BLAST analysis of the Expressed Sequence Tags (EST) database identified partial sequences of the putative mouse (accession no. AA396503) and human (accession no. F12722) G β L homologues whose partial amino acid sequences were 96 and 93% identical to rat G β L, respectively. The mouse clone was subsequently obtained from the Integrative Molecular Analysis of Gene Expression (IMAGE) Consortium and the Washington University Genome Sequencing Center in St Louis, MO, USA, and was sequenced at the Johns Hopkins University DNA Analysis Facility. Thereafter, the full-length sequence was deposited in the GenBank database (accession no. AF237676). The open reading frame of the mouse cDNA clone is 98.5% homologous to rat G β L cDNA, and the predicted proteins are 99% identical. The partial human G β L EST clone was isolated serendipitously by the generation of a transcriptional map for exonic regions adjacent to the polycystic kidney disease 1 gene and was mapped to human chromosome 16p13.3 (Burn *et al.* 1996). Screening of the human genome database identified the remaining portion of the human G β L open reading frame as well as the surrounding intronic and promoter sequences (GenBank accession no. AC009065). The predicted amino acid sequence of this putative homologue is 96.6% identical to rat G β L overall. In fact, the carboxy-terminal 204 residues of both proteins are indistinguishable. These data strongly suggest that the G β L homologues are extremely well conserved among mammals. BLAST analysis also identified a yeast (*Saccharomyces cerevisiae*) cDNA sequence that encodes a basic protein named POP3, whose mass is 22.6 kDa. Although slightly smaller than G β L, the predicted proteins are 60.4% similar and 44% identical overall. Moreover, these homologies are not limited to the WD-40 repeating motifs, but are evenly distributed throughout.

G β L mRNA tissue distribution

Northern blot analysis (Fig. 2) using a radiolabeled cDNA probe (Fig. 1) identified a 4 kb transcript in most tissues and an additional 2 kb transcript in testis and in CHO cells (data not shown). G β L mRNA was expressed abundantly in all tissues assayed, but was highest in testis and brain followed (in decreasing order) by lung, heart, kidney, skeletal muscle, spleen and liver. Additionally, RT-PCR (data not shown) showed that G β L was expressed in rat

Figure 2 Tissue distribution of G β L transcripts. A northern blot of poly(A)⁺ RNA (2 μ g/lane) from the indicated rat tissues was hybridized with a G β L-specific radiolabeled probe (Fig. 1) as described in the Materials and Methods. RNA size standards are indicated on the right (kb).

epididymal, abdominal and brown fat, small intestine, pancreas, 3T3-L1 fibroblasts and adipocytes, L6-myotubes and in rat insulinoma (RIN1046–38) cells.

Western blotting and immunoprecipitation of G β L

Antiserum against G β L (α G β L) was characterized by immunoprecipitating the antigenic peptide that was cross-linked to BSA (BSA-pep). The addition of excess peptide during the immunoprecipitation reaction displaced much of the BSA-pep within the immunoprecipitate (Fig. 3A). Therefore, α G β L specifically recognizes the antigenic peptide. Antiserum against the V5 epitope (α V5) and α G β L both recognized single proteins of 42 kDa in HEK 293 cells overexpressing a carboxy-terminal epitope-tagged G β L/V5/His fusion protein (Fig. 3B), indicating that G β L migrates slightly slower than its predicted mass of 36 kDa. This band was not recognized by preimmune serum (data not shown). In addition, western blotting of α G β L immunoprecipitates from crude membrane and cytosolic fractions indicated that the 42 kDa epitope-tagged G β L is located predominantly within the cytosol (Fig. 3C, left panel). Several bands within the immunoprecipitates were recognized by α V5-HRP in addition to the 42 kDa band that represents G β L. These additional bands were not detected during probing of whole-cell lysates (Fig. 3C, middle panel); they were detected, however, during probing of α G β L alone that was not used to immunoprecipitate (Fig. 3C, right panel). Thus, these additional bands are due to the cross-reaction of α V5-HRP to α G β L.

Insulin regulation of G β L production

The insulin receptor and G β L both appear to be similarly distributed in many different tissues; therefore, the ability of insulin to regulate G β L production was investigated. G α q/11, cG β and G β L protein levels were measured in

Figure 3 Western blotting and immunoprecipitation studies. (A) 7.5 and 75 pmol of BSA alone or BSA-conjugated antigenic peptide (BSA-pep) were immunoprecipitated with α G β L in the absence or presence (XS pep) of antigenic peptide. Immunoprecipitates were analyzed by Western blotting with α G β L, as described in the Materials and Methods. (B) Western blotting of whole-cell lysates from HEK 293 cells overexpressing an epitope-tagged (V5/His) G β L. The membrane was first probed with α V5-HRP (left) then stripped and reprobed with α G β L (right). (C) Crude cytosolic (cyto) and membrane (mem) fractions from transfected cells were immunoprecipitated with α G β L and the precipitated proteins were analyzed by Western blotting with α V5-HRP (left panel). Whole-cell lysates from these cells (middle panel) as well as α G β L that was not used in an immunoprecipitation reaction (right panel) were also analyzed by Western blotting with α V5-HRP. The data presented in the latter two panels indicate that the 30–40 kDa bands present in the left panel are due to the cross-reaction of α V5-HRP to the immunoprecipitating immunoglobulin G, α G β L.

fully differentiated 3T3-L1 adipocytes that were stimulated with varying concentrations of insulin. Although the G α q/11 and cG β levels were unaffected by insulin, G β L 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 G β L mRNA values between treatment groups by semi-quantitative RT-PCR, the linear portion of the amplification curve for both G β L 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, G β L=32) and G β L transcript values were then normalized to those of actin. Insulin raised the G β L 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 G β L mRNA levels were similar to the changes in protein levels; thus, the ability of insulin to upregulate G β L production is not due to an increase in translational efficiency *per se*, but appears to be due either to increased G β L gene transcription or to increased transcript stability.

Discussion

G β L possesses many structural features that are characteristic of G β 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 G β L and any of the known G β proteins suggests that G β L, unlike many of the mammalian G β subunits, did not originate from the same ancestral gene and is therefore not a legitimate G β homologue. However, BLAST analysis did identify a yeast ribonucleoprotein, POP3, whose amino acid sequence is 60.4% similar and 44% identical to rat G β L. 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 G β L 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 glycerol 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

Figure 4 Insulin regulation of G β L production. (A) Fully differentiated 3T3-L1 adipocytes were serum-starved for 48 h prior to the addition of insulin. Whole-cell extracts were analyzed by Western blotting with α G α q/11, α cG β or α G β L (representative blots are shown). (B) The linear portion of the cycle titration curve for actin and G β L transcripts was determined as described in the Materials and Methods. (C) After 32 cycles, G β L band intensities were normalized to those of actin after 25 cycles. Different letters represent differences between any two groups, whereas identical letters represent the fact that there are no differences ($n=4$).

transcript-specific increase in RNase activity is prevented by actinomycin D, indicating that gene transcription is required. Since the structural similarities between G β L 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 G β L.

The ability of insulin to upregulate G β L protein and mRNA levels in 3T3-L1 adipocytes suggests that insulin is a potent regulator of G β L production and imply that G β L 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 G β L directly interacts with any of these known intermediates, the ability of insulin to raise G β L production implies that it is nevertheless important to the actions of insulin.

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