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# Alternative splicing, gene localization, and binding of SH2-B to the insulin receptor kinase domain

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**Abstract.** The SH2-B protein is an SH2-domain-containing molecule that interacts with a number of phosphorylated kinase and receptor molecules including the insulin receptor. Two isoforms of the SH2-B have been identified and have been proposed to arise through alternate splicing. Here we have identified a third isoform of the SH2-B protein, SH2-Bγ, that interacts specifically with the insulin receptor. This interaction required phosphorylation of residue Y1146 in the triple tyrosine motif within the activation loop of the IR kinase and is one of only two signaling molecules shown to interact directly with this residue of the insulin receptor kinase domain. The intron/exon structure of the SH2-B gene was determined. Alternate splice sites utilized to generate the different isoforms of the SH2-B protein were identified in the 3′ end of the SH2-B gene immediately downstream of the exon encoding the core of the SH2 domain. Additionally, the chromosomal location of the SH2-B gene was determined to be the distal arm of mouse Chromosome (Chr) 7 in a region linked to obesity in mice.

## Introduction

The characterization of signaling pathways activated through engagement of the insulin receptor (IR) has greatly enhanced the understanding of molecular mechanisms involved in the intracellular pathways utilized by a number receptor systems. Indeed, the specific molecular events triggered by activation of the IR kinase that lead to the induction of signaling pathways are similar in a wide range of receptor systems (Cheatham and Kahn 1995; White and Kahn 1994). These molecular events include the phosphorylation tyrosine residues in the IR cytoplasmic domain and binding of these phosphotyrosines by specific signaling proteins. These signaling molecules include adapter molecules that act as molecular links between the IR and downstream signaling effector molecules. In particular, the adapter molecules IRS-1 and IRS-2, which are substrates of the IR kinase, have been shown to be critical to the activation of multiple signaling pathways by the IR as well as by more divergent receptors, such as the receptor for interleukin-4 and other cytokines (Keegan et al. 1994; Myers et al. 1994; Sun et al. 1991, 1995; Wang et al. 1993). Phosphorylation of the IRS-1 and IRS-2 molecules by the IR kinase and the interaction of phospho-IRS-1/IRS-2 with additional signaling molecules are critical to the activation of the Ras/MAPK and PI-3-kinase signaling pathways that are involved in a number of cellular responses to insulin (Backer et al. 1992; Myers et al. 1992; Sun et al. 1993). However, activation of additional pathways by the IR that contribute to certain physiological responses, such as glucose uptake, could involve as yet uncharacterized signaling molecules (Isakoff et al. 1995; Morris et al. 1996).

The yeast two-hybrid system has been demonstrated to be a highly effective method for identifying signaling molecules that interact with the IRβ cytoplasmic domain (He et al. 1995, 1998; O'Neill et al. 1994; Riedel et al. 1997; Wang and Riedel 1998). The success of this method has been due in part to the ability of the IRβ cytoplasmic tail to autophosphorylate when expressed as a bait molecule in yeast, thus enabling it to interact with molecules containing Src-homology 2 (SH2) and phosphotyrosine-binding (PTB) motifs (O'Neill et al. 1994, 1996). Interactions of the IRβ with a number of important SH2 and PTB-domain signaling molecules including Shc, Grb10, the p85 subunit of PI-3-kinase, as well as IRS-1 and IRS-2, have been characterized with this system (Gustafson et al. 1995; Hansen et al. 1996; He et al. 1996; O'Neill et al. 1994, 1996). Interactions with previously uncharacterized proteins have also been reported (Riedel et al. 1997). One of the novel IRβ-interacting proteins identified with this system, initially termed SH2-B, was first reported to interact with the phosphorylated cytoplasmic domain of the high affinity receptor for IgE (FcεRI) and contained pleckstrin homology (PH) and an SH2-domains (Osborne et al. 1995). Two isoforms of the mouse SH2-B protein (termed PSM-1 and PSM-V) were found to interact with the IRβ in the yeast two-hybrid system (Riedel et al. 1997; Wang and Riedel 1998). These proteins were identical except for sequences in the C-termini and thus were hypothesized to result from alternate splicing (Riedel et al. 1997). A C-terminal truncated isoform of SH2-B, termed SH2-Bβ, was identified on the basis of its ability to interact with the tyrosine kinase Jak2 and was identical to one of the aforementioned IRβ-binding SH2-B proteins (PSM-V) (Rui et al. 1997).

Additional proteins that are structurally related to SH2-B have been identified. One such protein, termed APS, is highly related to SH2-B in both the C-terminal SH2 domain (80% identity) and the PH domain (58% identity); the N-terminal region is less similar but still exhibits significant sequence homology (33% identity) (Yokouchi et al. 1997). APS was demonstrated to be phosphorylated in response to B cell receptor engagement and co-precipitated with the adapter Grb2 (Yokouchi et al. 1997). In addition, Shc appeared to interact constitutively with APS (Yokouchi et al. 1997). A second SH2-B-related protein, Lnk, was expressed primarily in lymphoid tissue and was phosphorylated in response to T cell receptor cross-linkage (Huang et al. 1995). Lnk lacks N-terminal sequences found in the SH2-B protein, but these proteins have a high degree of identity from midway through the PH do-

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The nucleotide sequence and mapping data reported in this paper have been submitted to Genbank (accession number AF074329) and to the Mouse Genome Informatics database (accession number 1201407).

main to the C-terminus; the PH and SH2 domains of Lnk and SH2-B are 40% and 72% identical, respectively (Huang et al. 1995). Lnk also has been shown to interact with signaling molecules such as Grb2, phospholipase Cγ, and PI-3-kinase, after T cell activation (Huang et al. 1995). Together, these data suggested that APS and Lnk may act as adapter molecules in a variety of signaling pathways.

The high degree of similarity between SH2-B, APS, and Lnk suggests that these proteins represent a new family of structurally related adapter molecules. Here we have identified a novel member of the SH2-B family of proteins that arises from alternate splicing of the SH2-B gene transcript. This novel isoform of SH2-B, termed SH2-Bγ, interacts with the IR kinase domain and requires residue Y1146 in the phosphorylated triple tyrosine motif for this interaction. Additionally, the alternate splice sites that are used to generate isoforms of SH2-B have been characterized, and the genetic location of the SH2-B gene has been identified.

## Materials and methods

*Yeast two-hybrid system.* The yeast strain EGY48 (*trp1, ura3, his3, leu2*) and yeast expression vectors (provided by the laboratory of Roger Brent, Harvard Medical School) have been previously described (Golemis and Brent 1992). Construction of a IRβ bait containing the cytoplasmic domain (pLexA.IR.cyto, referred to as the IRβ bait) and kinase or tyrosine mutant forms of this IRβ bait have been detailed elsewhere (Gustafson et al. 1995; O'Neill et al. 1994, 1996). Interactions between the SH2-Bγ protein and IRβ baits were determined through the expression of the β-galactosidase reporter gene (Estojak et al. 1995). The SH2-Bγ-fusion protein used in the yeast two-hybrid interaction studies included the SH2 domain and sequences C-terminal to the SH2 domain of SH2-Bγ (encoded by nucleotides 1374–2049 of the SH2-Bγ cDNA, GenBank (AF074329) fused to the VP16 transcriptional activation domain. β-Galactosidase activity was analyzed through colony color or solution assays as described (He et al. 1996; O'Neill et al. 1994).

*Yeast two-hybrid library screen.* A mouse pre-B cell cDNA library in the yeast expression vector JG4-5 was a kind gift of the laboratory of Zheng Sheng Ye (Rockefeller University). This library was screened with the IRβ bait according to described protocols (Gyuris et al. 1993; Zervos et al. 1993). Isolated clones were sequenced by Paragon Biotech Inc. (Baltimore, Md.), and full-length clones were obtained by screening a mouse liver cDNA library (Stratagene, La Jolla, Calif.) with partial cDNAs obtained from the yeast library screen.

*Northern blot analysis.* Multi-tissue Northern blots (Clontech, Palo Alto, Calif.) were probed with a 2-kb fragment encompassing the 3′ end of the SH2-Bγ cDNA or a human β-actin probe by standard protocols. Probes were labeled with a random primer oligolabeling kit (Pharmacia Biotech, Piscataway, N.J.).

*Mapping of intron and exon boundaries.* A 6.1-kb PCR fragment was amplified from C57BL6/JEi genomic DNA with eLONGase high-fidelity polymerase (GibcoBRL/Life Technologies, Gaithersburg, Md.) and the following primers from the 5′ UTR and 3′ UTR of the SH2-B cDNA: 5′ CATTGGACCTATGATGCAAGGTCTTTAAAGGG (forward) and 5′ CTTAAGGCTGGGAGCAGGAAAATGG (reverse) [annealing temperature 68°C]. Multiple sequencing primers derived from the SH2-B cDNA sequence were used to directly sequence genomic PCR fragments. Intron/exon boundaries were inferred from comparison of the cDNA and genomic sequences. Splice donor and acceptor sites were determined by comparison with known splice consensus sequences and with the BCM Gene Finder program (available on the World Wide Web at http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html).

*GST fusion protein precipitations and Western blot analyses.* A GST-fusion protein expression vector containing the SH2 domain of SH2-Bγ (SH2-Bγ SH2-GST) was produced by subcloning a *Eco*RI-*Pst*I restriction fragment (nucleotides 1364–1850 of the SH2-Bγ cDNA, GenBank AF074329) into pGEX-5X-2 (Pharmacia Biotech, Uppsala, Sweden). The GST-fusion protein was expressed in bacteria and purified with glutathione-agarose beads as described (Ausubel et al. 1993). The beads that contained immobilized fusion protein were then incubated with cell lysates derived from CHO.T cells (which overexpress the IR) prior to or after insulin stimulation (10 min, 100 nM). Lysates were prepared by lysis for 30 min on ice in 50 mM HEPES (pH 7.6), 1% Triton X-100, 1 mM EGTA, 10 mM NaF, 20 mM sodium pyrophosphate, 1 mM PMSF, 1 mM sodium orthovanadate, and 10 μg/ml of aprotinin and leupeptin followed by spinning at 10,000 *g* for 10 min to remove insoluble material. The resulting lysate supernatants were incubated with the immobilized GST proteins (~5 μg) for 4 h to overnight. After extensive washing with 50 mM HEPES (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, the proteins that coprecipitated with the SH2-Bγ SH2-GST or control GST proteins were analyzed by SDS-PAGE, followed by immunoblotting with either anti-IR (IR-CT1) (a gift from Ken Siddle), anti-phosphotyrosine antibodies (PY20) (Transduction Labs, Lexington, KY) or anti-IRS-1 (CT-1 Upstate Biotechnology, Lake Placid, NY).

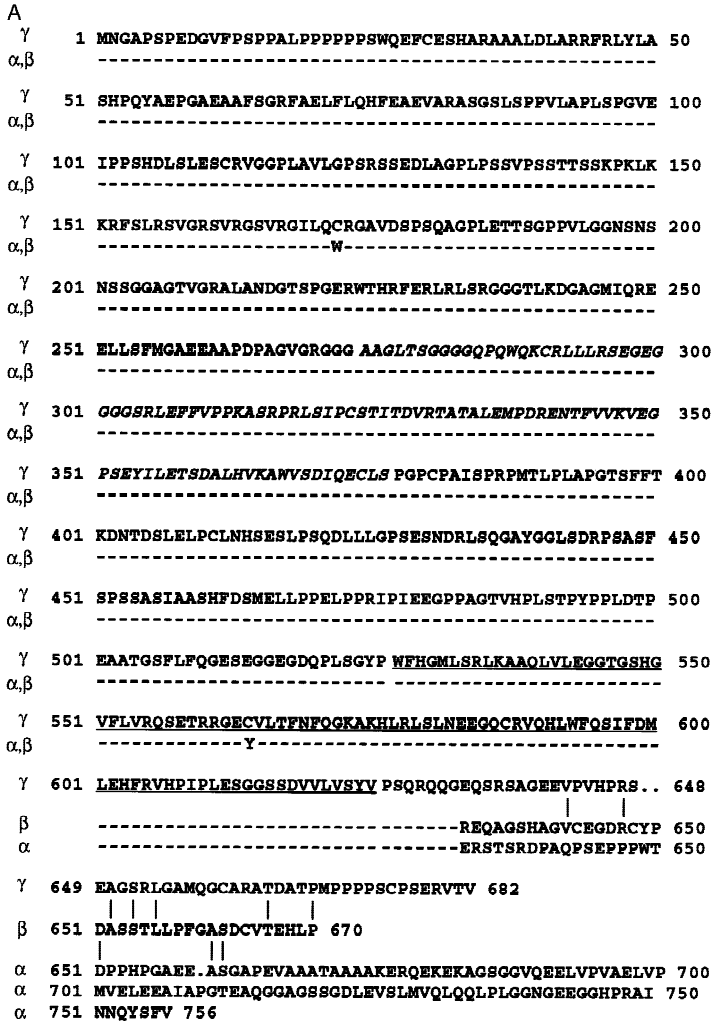
*Insulin receptor kinase gel shift assay.* The tyrosine kinase domain of the IR (IRK) was purified from baculovirus-infected Sf-9 cells and autophosphorylated as described (Hubbard 1997). Approximately equal amounts of purified unphosphorylated IRK, bis-phosphorylated IRK (predominantly pY1150/1151) for which auto-dephosphorylation had occurred (Wei et al. 1995), and tris-phosphorylated IRK (pY1146/1150/1151) were mixed, to which was added the SH2-Bγ SH2-GST fusion protein purified on glutathione-agarose beads. Samples were loaded onto a native (nondenaturing) 20% homogeneous polyacrylamide PhastGel and electrophoresed for a total of 132 volt-hours on a PhastSystem (Pharmacia Biotech, Piscataway, NJ).

*Genetic mapping of the SH2-B gene.* Mapping of the SH2-B gene was done with assistance from the Jackson Laboratory backcross mapping service,with the [(C57BL6/JEi × SPRET/Ei)$F_1$ × SPRET/Ei] (BSS) backcross panel. A 425-bp PCR fragment was amplified from nucleotide 2571–2997 of the SH2-B gene (number according to Genbank AF020526) with the following primers: 5′ TACAGCTCCAGCAGCTACCACTAGG (forward) and 5′ ACAACAACCTTAATATAACTGACAGCCC (reverse) [annealing temperature 67°C]. A sequence polymorphism in the SPRET/Ei allele resulted in the loss of a *Bsp*HI restriction site at nucleotide 2806 in this region. PCR fragments generated from the 94 animals in the BSS backcross were restricted with *Bsp*HI and analyzed by gel electrophoresis on 2% agarose gels. The 425-bp PCR fragment from the C57BL6/JEi allele was restricted into 235-bp and 190-bp fragments, while the SPRET/Ei allele remained undigested.

## Results

The yeast two-hybrid system was used to identify additional molecules that interact with the phosphorylated IR (Gyuris et al. 1993; O'Neill et al. 1996; Zervos et al. 1993). A bait molecule consisting of the cytoplasmic domain of the IRβ was used to screen a pre-B cell cDNA library as described (O'Neill et al. 1996). Phosphorylation of the IRβ cytoplasmic tail was detected as previously reported (K. Nelms, unpublished data; Gustafson et al. 1995; O'Neill et al. 1996). Approximately $5 \times 10^5$ independent clones were screened, from which 16 cDNAs encoding proteins capable of interacting specifically with the IRβ bait were identified as measured by induction of LacZ reporter expression. These cDNAs were isolated and sequenced, and a number were found to be previously described genes for proteins that interact with the IR (Gustafson et al. 1995; Tartare-Deckert et al. 1996). These included Vav, Grb2, and the p85 regulatory subunit of phosphatidylinositol-3-kinase (K. Nelms, unpublished data). Several clones also contained unidentified sequences. One of these, a 2.1-kb cDNA, encoded a protein that interacted very strongly with the IRβ bait and was further characterized.

This 2.1-kb clone represented a partial cDNA and was thus used to screen a liver cDNA library. Overlapping clones representing a 3.1 kb full-length cDNA were isolated, sequenced, and found to be highly related to the murine homolog of the rat SH2-B protein [termed PSM-1 according to (Riedel et al. 1997)] and



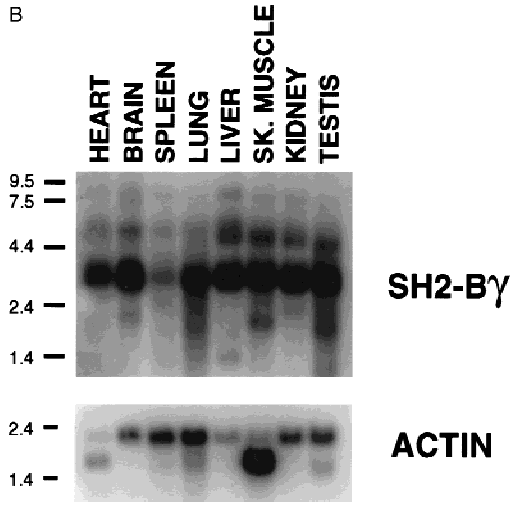



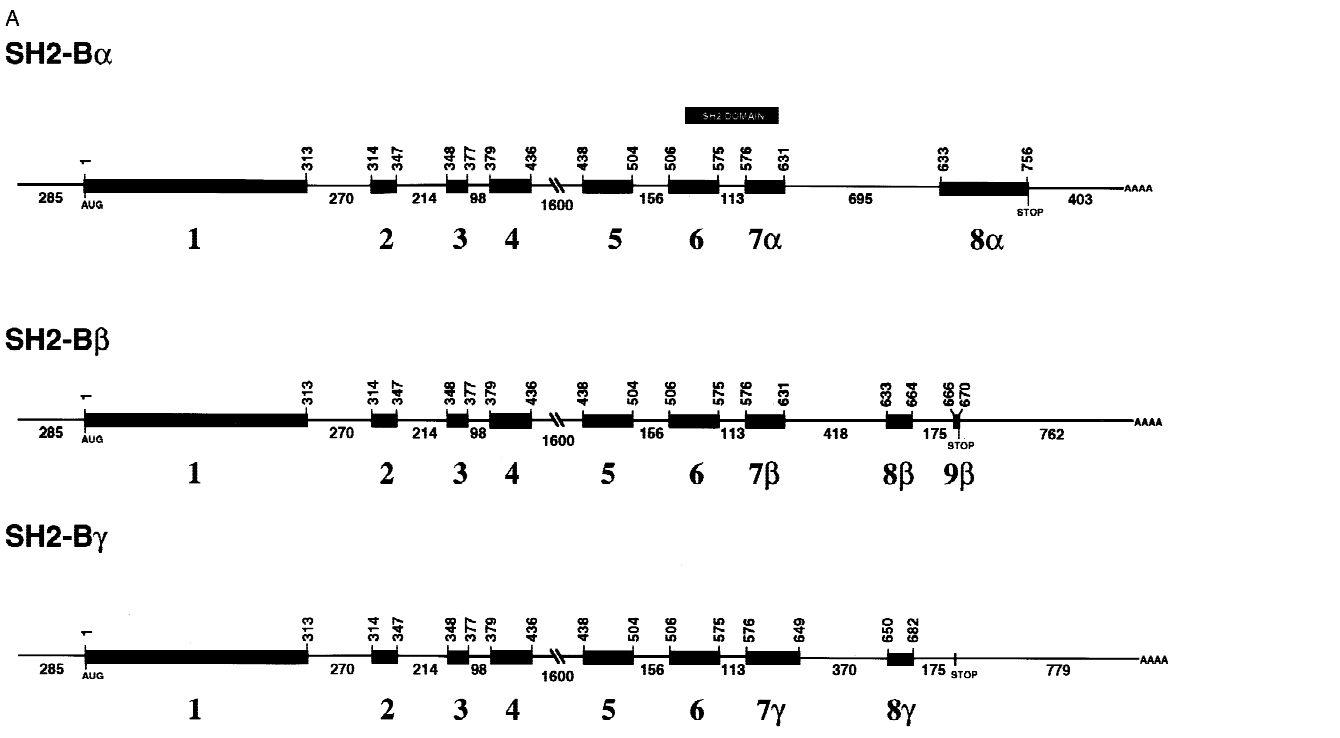

**Fig. 1.** SH2-Bγ is a C-terminal variant of the SH2-B gene and is highly expressed in different tissues. (A) The protein sequence of SH2-Bγ is identical to that of SH2-Bα and SH2-Bβ through residue 625 but diverges at the C-terminus. The SH2-Bγ (γ), SH2-Bα (α), SH2-Bβ (β) amino acid sequences are indicated. The pleckstrin homology domain is in italics, and the SH2 domain is underlined. Amino acid differences at positions 172 and 564 are due to strain-specific polymorphisms. (B) Northern analysis of SH2-Bγ (upper) and β-actin (lower) gene expression in different mouse tissues.

SH2-Bβ (also termed PSM-V), but differed from these proteins at the C-terminal end (Fig. 1A). Thus, we hypothesized that this cDNA encoded a third splice variant of the SH2-B gene and refer to it as SH2-Bγ. We also propose that the other isoforms, SH2-B/PSM-1 and SH2-Bβ/PSM-V, be referred to as SH2-Bα and SH2-Bβ, respectively. The N-terminal 632 amino acids of SH2-Bα, SH2-Bβ, and SH2-Bγ are more than 99% identical and contain the PH and SH2 domains (Fig. 1A). The two residues that differ in this region are due to mouse strain-specific polymorphisms (K. Nelms, unpublished observations). In contrast, the divergent C-termini exhibited very limited homology (Fig. 1A).

Expression of the SH2-Bγ gene was examined by Northern analysis with a probe that contained the unique 3′ end of the SH2-Bγ cDNA. The SH2-Bγ mRNA was expressed widely and at high levels in all tissues examined except the spleen, where expression was detectable but significantly lower than in other tissues (Fig. 1B). The expression pattern of SH2-Bγ mRNA differed from the expression pattern previously reported for the SH2-Bα mRNA. In contrast to the SH2-Bγ message, SH2-Bα mRNA was shown to be expressed at high levels in the brain and liver, but at low or undetectable levels in other tissues including lung, kidney, testis, and spleen (Riedel et al. 1997). Additional bands detected in this Northern analysis may reflect cross-hybridization of the SH2-Bγ probe with RNA encoding related proteins. Indeed, APS has been shown to be 80% identical in the SH2 domain and 58% in the PH domain to SH2-Bα (Yokouchi et al. 1997).

The sequence of different isoforms of the SH2-B protein suggested a pattern of alternate splicing in the 3′ end of the transcript. To determine how the SH2-Bα, SH2-Bβ, and SH2-Bγ transcripts are produced, the intron/exon structure of the SH2-B gene was determined. A 6.1-kb genomic fragment was amplified by high-fidelity PCR with primers from the 5′ and 3′ untranslated regions of the SH2-Bγ transcript. The resultant PCR product was sequenced with primers from within the cDNA sequence, and the intron/exon boundaries for the SH2-Bα, SH2-Bβ, and SH2-Bγ genes were determined by comparison of the genomic and cDNA sequences (Fig. 2A). Exons 1 through 6 of the SH2-Bα, SH2-Bβ, and SH2-Bγ transcripts were found to be identical. Introns within this region ranged in size from 98 to 1600 nucleotides. Exons 6 and 7 contained sequences encoding the SH2 domain (Fig. 2A). The first 168 nucleotides of exon 7 in the SH2-Bα, SH2-Bβ, and SH2-Bγ transcripts (7α, 7β, and 7γ, respectively) are identical and encode most of the C-terminal end of the SH2 domain (Figs. 1A and 2A). The use of alternate splice sites at the 3′ end of exon 7 results in the different SH2-B transcripts.

The genomic sequence of the SH2-B gene from exon 7 through the transcription stop site was examined, and the alternate splice sites were mapped (Fig. 2B and Table 1). The SH2-Bα and SH2-Bβ transcripts utilized the same splice donor site at the 3′ end of exon 7. The use of alternate splice sites beyond the 3′ end of exons 7α and 7β results in C-terminal sequence differences between SH2-Bα and SH2-Bβ. The SH2-Bα transcript is spliced from the 3′ donor site of exon 7α to an acceptor site 695 nucleotides downstream at the 5′ end of a 369-nucleotide exon, 8α, unique to the SH2-Bα transcript (Fig. 2A and 2B). In contrast, the SH2-Bβ transcript is spliced from the 3′ donor site of exon 7β to an acceptor site 418 nucleotides downstream at the 5′ end of a 93-nucleotide exon, 8β (Fig. 2A and 2B). The 3′ end of exon 8β is




B

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CACCTGCGCTTGTCACTAAATGAGGAGGGACAGTGCCGGGTCCAACATCTGTGGTTCCAGTCCATTTTCGATATGCTTGAGCACTTCCGGGTGCACCCCATCCCTCTGGAGTCTGGAGGC
H  L  R  L  S  L  N  E  E  G  Q  C  R  V  Q  H  L  W  F  Q  S  I  F  D  M  L  E  H  F  R  V  H  P  I  P  L  E  S  G  G  -

TCCAGTGATGTTGTCCTTGTCAGCTATGTGCCCTCCCAGCGGCAGCAGG-GTGAGCAGAGCAGGTCTGCAGGGGAGGAGGTGCCCGTGCACCCAAGAAGTGAG-GTgtgtgtgccaggaagac
S  S  D  V  V  L  V  S  Y  V  P  S  Q  R  Q  Q  G    E  Q  S  R  S  A  G  E  E  V  P  V  H  P  R  S  E

ggggttggggagggtggaggaaaggccctgttagtggggagcagggcagtcaggtacctgggaggggcagtggagagctgggtgctgcattcccattccattggacccctccgcgccatcat

tgtctgtctcctggacccatcctcctcagccttgtctttggccttttgggggattcatggcctgatcccctccttcctcccttgatgtctgatgtccctgtctgatctctcactttccccc

acccaccatcccatctgtccccacgttgcccctcccctcccctccccAG-GCCGGGAGCAGGCTGGGAGCCATGCAGGGGTGTGCGAGGGCGACCGATGCTACCCCGATGCCTCCTCCAC
                                                  G  R  E  Q  A  G  S  H  A  G  V  C  E  G  D  R  C  Y  P  D  A  S  S  T
                                                  A  G  S  R  L  G  A  M  Q  G  C  A  R  A  T  D  A  T  P  M  P  P  P  P

CCTCCTGCCCTTCGGAGCGAGTGACTGTGT-GTaagtgtggtcctctcaccaccgcccatgattcatcttccatggaggggggttgctcaggagatgggacatgggggagatagcacatgg
L  L  P  F  G  A  S  D  C  V
S  C  P  S  E  R  V  T  V  *

ctccttggggaggacaagcaaaggggaggtcgccataagaactcacttccttccacaagcaatgtcttctcaccattctcatccAG-AACGGAGCACCTCCCGTGACCCAGCCCAGCCCTC
                                                                                        T  E  H  L  P  *
                                                                                        E  R  S  T  S  R  D  P  A  Q  P  S

TGAACCCCCTCCATGGACAGATCCCCCACATCCTGGGGCAGAAGAGGCGTCGGGGGTGCCAGAAGTTGCGGCAGCCACAGCCGCAGCAGCCAAAGAGAGGCAAGAGAAAGAGAAAGCGGG
E  P  P  P  W  T  D  P  P  H  P  G  A  E  E  A  S  G  V  P  E  V  A  A  A  T  A  A  A  A  K  E  R  Q  E  K  E  K  A  G

CAGTGGAGGGGTCCAGGAAGAGCTGGTCCCCGTGGCTGAGCTGGTCCCCATGGTTGAATTGGAAGAGGCCATAGCACCAGGCACTGAGGCTCAGGGTGGTGCTGGCTCTAGTGGGGACTT
S  G  G  V  Q  E  E  L  V  P  V  A  E  L  V  P  M  V  E  L  E  E  A  I  A  P  G  T  E  A  Q  G  G  A  G  S  S  G  D  L

GGAGGTGTCCCTAATGGTACAGCTCCAGCAGCTACCACTAGGGGGCAACGGAGAAGAAGGGGGTCACCCCCGAGCCATTAATAATCAGTACTCATTTGTGTGAGATACCTGCCCACCCTC
E  V  S  L  M  V  Q  L  Q  Q  L  P  L  G  G  N  G  E  E  G  G  H  P  R  A  I  N  N  Q  Y  S  F  V  *

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aaagggtttatctcagttccatcaaaa
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**Fig. 2.** Alternate splicing in the 3′ end of the SH2-B gene results in the expression of the SH2-Bα, SH2-Bβ, and SH2-Bγ forms. (A) Intron/exon structure of the SH2-B gene. Splicing of each SH2-B gene transcript is identical through exon 6. Use of alternate splice sites at the 3′ end of exon 7 results in the α, β, or γ forms. Intron/exon structure of each form (α, β, or γ) is indicated. (B) Genomic sequence of the 3′ end of the SH2-B gene. Identical protein sequences coded by exon 7 α, β, and γ are underlined, Protein sequences unique to the γ form are in italics. Sequences unique to the β form are in standard type. Sequences unique to the α form are in underlined italics. Stop codons for each form are indicated (*). Splice sites are indicated by dashes (-). Intron sequences are in lowercase, coding sequences are in uppercase.

spliced to the same acceptor site used at the 5′ end of exon 8α of the SH2-Bα transcript, but the translational reading frame is shifted relative to the SH2-Bα transcript, resulting in a novel exon (9β) encoding 5 amino acids and a translation stop (Fig. 2A and 2B). All splice sites identified matched the consensus sequence for mammalian splice donor and acceptor sequences (Table 1; Green 1991).

The SH2-Bγ transcript results from the use of a splice donor site 53 nucleotides 3′ to the splice donor site used in exons 7α and 7β of the SH2-Bα and SH2-Bβ transcripts, respectively (Fig. 2B). This alternate site is spliced to the same donor and acceptor sites used in exons 8β and 9β of the SH2-Bβ transcript (Table 1). However, the use of the alternate splice donor site at the 3′ end of exon 7γ results in a frameshift and an exon, 8γ, that encodes a

**Table 1.** Splice site sequences used in the α, β and γ forms of SH2-B.

| Exon (donor site) | | Intron (bp) | Exon (acceptor site) | |
|---|---|---|---|---|
| | **K** | | | |
| 1 | CCC AAG \| gtgagc | 270 | 2 | gcgtag \| GCG TCC |
| | **K** | | | |
| 2 | GTT AAG \| gtagga | 214 | 3 | ctgtag \| GTA GAA |
| | **P** | | | |
| 3 | C CCC GG \| gtaaga | 98 | 4 | ttgtag \| A CCC TG |
| | **Q** | | | |
| 4 | CG CAG G \| gtaagg | 1600 | 5 | tttcag \| GA GCT T |
| | **T** | | | |
| 5 | CC ACA G \| gtattg | 156 | 6 | ctgaag \| GA TCG T |
| | **K** | | | |
| 6 | GCC AAG \| gtgagt | 113 | 7 α, β, γ | cctcag \| CAC CTG |
| | **Q** | | | |
| 7 α | AG CAG G \| gtgagc | 695 | 8 α | atccag \| AA CGG A |
| | **Q** | | | |
| 7 β | AG CAG G \| gtgagc | 418 | 8 β | ccccag \| GC CGG G |
| | **C** | | | |
| 8 β | C TGT GT \| gtaagt | 175 | 9 β | atccag \| A ACG GA |
| | **E** | | | |
| 7 γ | AGT GAG \| gtgtgt | 370 | 8 γ | ccccag \| GCC GGG |
| | **V** | | | |
| 8 γ | CT GTG T \| gtaagt | 175 | STOP | atccag \| AA CG GA |

Exon numbers and α, β or γ designations are as in Fig. 2. Codon usage in the donor splice site is indicated, as are the intron sizes (bp).

sequence unique to SH2-Bγ. Splicing at the 3′ end of exon 8γ results in the formation of a translation termination codon (Fig. 2B and Table 1).

We wished to determine what region(s) of the IRβ cytoplasmic tail was required for its interaction with SH2-Bγ. Binding analyses were performed in the yeast two-hybrid system by co-expression of the SH2-Bγ protein with mutants of the IRβ bait molecule. Molecular interactions were indicated by the activity of β-galactosidase expressed from the LacZ reporter gene as described (He et al. 1996; O'Neill et al. 1994). The interaction of wild-type and mutant IRβ bait molecules with other signaling molecules in the yeast two-hybrid system has previously been demonstrated to reflect the ability of these molecules to interact in vivo (Gustafson et al. 1995; O'Neill et al. 1994; Sun et al. 1992; Tartare-Deckert et al. 1996).

SH2-Bγ interacted strongly with the wild-type (wt) IRβ bait molecule (Fig. 3A). Mutation of Lys1018 to Ala in the ATP binding pocket (K1018A) eliminated IRβ kinase function and blocked the interaction with SH2-Bγ, indicating that the kinase activity of the IRβ bait was required for its interaction with SH2-Bγ (Fig. 3A). Similarly, independent mutations in two membrane proximal tyrosine residues, Y953 and Y960 (Y953F and Y960F), did not diminish binding of SH2-Bγ to the IRβ bait molecule (Fig. 3A). The interaction of SH2-Bγ was compared with that of the adapter molecules Shc and IRS-1. In contrast to SH2-Bγ, mutation of Y960 completely blocked the interaction of Shc and IRS-1 with the IRβ bait (Fig. 3A). These interactions have been shown to be mediated by the PTB domains of IRS-1 and Shc that specifically recognize phosphorylated Y960 (Gustafson et al. 1995; O'Neill et al. 1994). Mutation of Y953 did not affect the IRS-1 or Shc interactions (Fig. 3A). Additionally, deletion of the C-terminal 30 amino acids containing two tyrosine residues, Y1316 and Y1322, did not lessen the interaction of SH2-Bγ, Shc, or IRS-1 with the IRβ bait.

Three tyrosine residues in the IRβ kinase domain, Y1146, Y1150, and Y1151, are also phosphorylated upon kinase activation. The Y1150/1151 residues have been shown to be required for full IRβ kinase activity (Ellis et al. 1986). The function of Y1146 is less clear. Mutation of Y1146 (Y1146F) completely blocked the interaction of SH2-Bγ with the IRβ bait molecule, but did not affect the interaction of Shc or IRS-1 with the mutant bait (Fig. 3A). This strongly suggests that Y1146 is a site of specific interaction of SH2-Bγ with the IRβ cytoplasmic domain. Tandem mutation of Y1150 and Y1151 (Y1150/1151F) diminished the interaction of SH2-Bγ, Shc, and IRS-1 with the IRβ bait (Fig. 3A). This may either reflect the role of these residues in full kinase function or suggest a role in the direct molecular interaction with these signaling molecules.

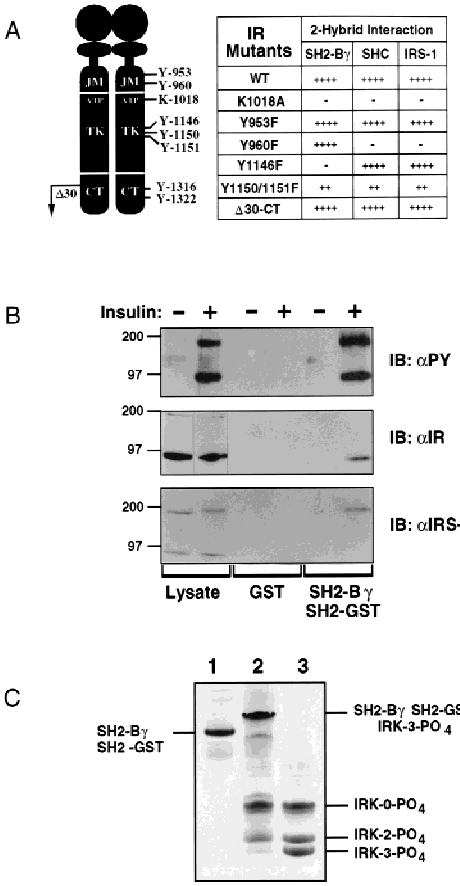


**Fig. 3.** SH2-Bγ binds to phosphorylated Y1146 in the triple tyrosine motif of the insulin receptor β kinase domain. (A) Analysis of the interaction of SH2-Bγ with wild-type (WT) or mutant forms of the IRβ cytoplasmic tail (IR) in the yeast two-hybrid system. Interactions of SH2-Bγ with WT, an ATP binding mutant (K1018A), juxtamembrane (JM) tyrosine mutants (Y953F and Y960F), tyrosine kinase (TK) domains mutants (Y1146F and Y1150/1151F), and a C-terminal deletion mutant (IRΔ30) lacking two tyrosine residues (Y1316/1322) were measured. Strong (++++), moderate (++), or no (−) interaction is indicated by β-galactosidase reporter gene expression. The SH2-Bγ interaction was compared with the interactions of the SHC and IRS-1 molecules with the IR mutants. (B) Interaction of an SH2 domain-GST fusion protein (SH2-Bγ SH2-GST) containing the SH2 domain and unique C-terminal sequences of SH2-Bγ with the IR and IRS-1 in CHO-IR cells. Precipitations with GST control or the SH2-Bγ SH2-GST were performed from total cell lysates (Lysate) before (−) and after (+) insulin stimulation. Precipitates were analyzed by immunoblotting (IB) with anti-phosphotyrosine (αPY, upper panel), anti-insulin receptor (αIR, middle panel), and anti-IRS-1 (αIRS-1, lower panel). (C) In vitro interaction between SH2-Bγ SH2-GST fusion protein and various phosphorylated forms of the IR kinase (IRK). Lane 1 contains SH2-Bγ SH2-GST fusion protein alone; lane 3 contains a mixture of unphosphorylated IRK (IRK-0-$PO_4$), bis-phosphorylated IRK (IRK-2-$PO_4$, predominantly pY1150/1151), and tris-phosphorylated IRK (IRK-3-$PO_4$, pY1146/1150/1151); and lane 2 contains a mixture of SH2-Bγ SH2-GST fusion protein and the various phosphorylated forms of IRK. Each band in lanes 1 and 3 contains approximately 1–3 μg of protein. Note that in lane 2, the IRK-3-$PO_4$ band is absent, but the IRK-0-$PO_4$ and IRK-2-$PO_4$ bands remain. A new, supershifted band is present which represents the SH2-Bγ SH2-GST/IRK-3-$PO_4$ complex.

The interaction of SH2-Bγ with the IRβ cytoplasmic tail was further investigated by analyzing the interaction of a GST-fusion

protein containing the SH2 domain of SH2-Bγ (SH2-Bγ SH2-GST) with cellular IR in co-precipitation experiments. CHO cells overexpressing the human IR were stimulated with insulin (100 nM, 10 min), and lysates were precipitated with either GST or SH2-Bγ SH2-GST. Total lysate and precipitates were analyzed by SDS-PAGE and Western blotting with anti-phosphotyrosine, anti-IR, or anti-IRS-1 (Fig. 3B). Two proteins of about 90 kDa and 180 kDa showed significant tyrosine phosphorylation in response to insulin stimulation in total lysates (Fig. 3B, upper panel). These proteins were precipitated with the SH2-Bγ SH2-GST but not the GST control fusion proteins. Immunoblotting with anti-IR and anti-IRS-1 indicated that the 97 kDa band was the IR and the 180 kDa band was IRS-1 (Fig. 3B, middle and lower panels). These data confirmed the results obtained in the yeast two-hybrid system and further indicated that the SH2 domain of SH2-Bγ could interact directly with cellular IR after insulin stimulation. These results also suggest that SH2-Bγ interacts with a complex containing IR and IRS-1. However, from this experiment it can not be ruled out that the SH2-Bγ SH2-GST interacts directly with phosphorylated IRS-1.

The requirement of Y1146 for interaction of SH2-Bγ with the IRβ cytoplasmic domain in the yeast two-hybrid system is only the second time that Y1146 has been shown to mediate an interaction between a signaling molecule and the IRβ; previously, interaction of the tyrosine phosphatase SHP-2 with the insulin receptor was shown to be inhibited by phosphopeptides containing Y1146 (Kharitonenkov et al. 1995). Therefore, the interaction of SH2-Bγ with the IRβ kinase domain was further analyzed in vitro by examining the interaction of the SH2-Bγ SH2-GST with different phosphorylated forms of the IR core kinase domain (IRK) containing residues 966–1271 (Wei et al. 1995). The order of autophosphorylation in vitro has been established for IRK (Wei et al. 1995). Either Y1150 (predominantly) or Y1146 is phosphorylated first, and Y1151 is phosphorylated last. During the later phase of the reaction, IRK begins to auto-dephosphorylate, with phosphorylated Y1146 (pY1146) hydrolyzed first, followed by either pY1150 or pY1151. The various phosphorylated forms of IRK are readily separated on non-denaturing polyacrylamide gels, with higher phosphorylated forms migrating more rapidly than lower phosphorylated forms (Fig. 3C, lane 3). Addition of the SH2-Bγ SH2-GST fusion protein to a mixture of unphosphorylated IRK, bis-phosphorylated IRK (pY1150/1151, obtained from the auto-dephosphorylation phase), and tris-phosphorylated IRK (pY1146/1150/1151) resulted in the mobility shift of only tris-phosphorylated IRK. This is further evidence that the SH2 domain of SH2-Bγ specifically interacts with pY1146 of the IRβ cytoplasmic domain.

To gain further insight into the potential role of SH2-B proteins in IR signaling pathways, we mapped the SH2-B gene using the The Jackson Laboratory interspecific backcross panel (C57BL/6JEi × SPRET/Ei)$F_1$ × SPRET/Ei called Jackson BSS. A polymorphism in the 3′ untranslated region of the SH2-B gene resulted in the loss of a *Bsp*HI restriction site in the *Mus spretus* allele. This polymorphism was used to type the 94 animals in the BSS panel. Comparison with other markers in the BSS backcross panel led to the localization of SH2-B gene to the distal arm of mouse Chr 7 (Fig. 4). This region of mouse Chr 7 has previously been determined to contain a locus involved in a multifactorial model of obesity in mice (Warden et al. 1993, 1995).

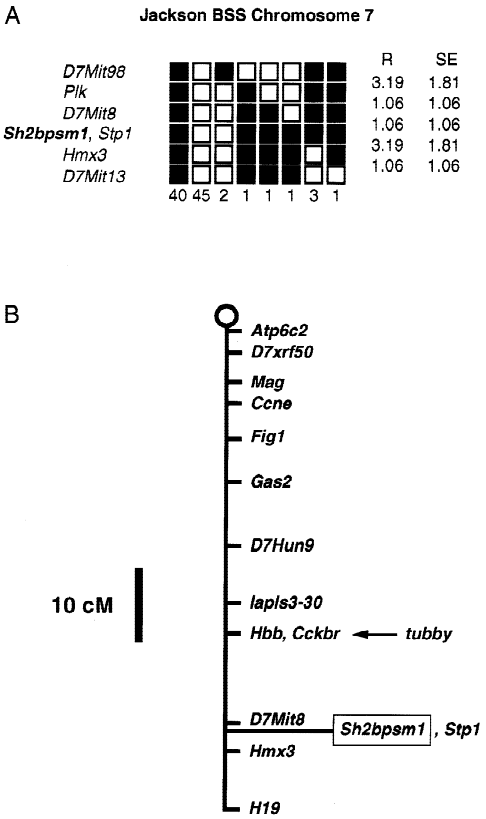


**Fig. 4.** The SH2-B gene maps to distal mouse Chr 7. (A) Haplotype figure from The Jackson BSS backcross showing part of Chr 7 with loci linked to SH2-B (Locus symbol *Sh2bpsm1*). Loci are listed in order, with the most proximal at the top. The black boxes represent the C57BL6/JEi allele, and the white boxes the SPRET/Ei allele. The number of animals with each haplotype is given at the bottom of each column of boxes. The percentage recombination (R) between adjacent loci is given to the right of the figure, with the standard error (SE) for each R. Raw data from The Jackson Laboratory were obtained from the World Wide Web address http://www.jax.org/resources/documents/cmdata. (B) Map figure from The Jackson Laboratory BSS backcross showing part of mouse Chr 7 containing the *Sh2bpsm1* locus and representative flanking markers. The region to which the *tubby* gene has been mapped is indicated by an arrow. The map is depicted with the centromere at the top. Relative distances between markers are indicated by a 10 cM scale bar.

## Discussion

The adapter protein SH2-B has been shown to be involved in the signaling pathways of a growing number of receptors including the FcεRI receptor, growth hormone receptor, interferon gamma receptor, and IR and belongs to a family of related molecules that includes APS and Lnk (Huang et al. 1995; Osborne et al. 1995; Riedel et al. 1997; Rui et al. 1997; Yokouchi 1997). Different isoforms of the SH2-B protein, which we have referred to as SH2-Bα and SH2-Bβ, were initially shown to interact with phosphorylated IR and Jak2 kinases and have been proposed to be produced through alternate splicing of the SH2-B gene transcript (Riedel et al. 1997; Wang and Riedel 1998; Rui et al. 1997). Here we have characterized a new isoform of the SH2-B protein, SH2-Bγ, that binds directly to the phosphorylated kinase domain of the IR and is also a product of alternate splicing.

We have mapped the intron/exon structure of the SH2-B gene and have identified alternate splice sites that are utilized to produce mature messenger RNA encoding the SH2-Bα, SH2-Bβ, and SH2-Bγ proteins. Differential use of splice sites in the 3′ end of the SH2-B transcript accounts for the C-terminal sequence differences between the SH2-Bα, SH2-Bβ, and SH2-Bγ isoforms. Although the same splice site at the end of exon 7 is used to produce the SH2-Bα and SH2-Bβ transcripts, additional sites are used in the SH2-Bβ transcript that are spliced out of the SH2-Bα transcript (Fig. 2B). The SH2-Bγ and SH2-Bβ transcripts differ only in the splice donor site used at the end of exon 7. Differential splicing in the SH2-Bα, SH2-Bβ, and SH2-Bγ transcripts leads to translation reading frame shifts and gives rise to the unique C-terminal amino acid sequences of these isoforms.

The differential splicing of genes can be regulated, and it will

be important to determine whether cellular context or specific stimuli result in the alternate splicing pattern of SH2-B gene transcripts (Green 1991; McKeown 1992). In this regard, it is significant that the expression pattern of the SH2-Bγ mRNA, as indicated by Northern analysis, differs from that reported for SH2-Bα (Osborne et al. 1995; Riedel et al. 1997). In particular, SH2-Bγ mRNA was expressed at high levels in all tissues examined except spleen, whereas SH2-Bα mRNA was expressed at comparable levels only in the brain and liver. This may reflect the fact that the SH2-Bγ probe used in the Northern analyses contained 3′ sequences unique to the SH2-Bγ isoform. Interestingly, the SH2-B-related genes encoding APS and Lnk also have distinct patterns of expression. Lnk is expressed specifically in hematopoietic tissue, whereas APS is expressed in various tissues and at high levels in Burkitt lymphoma cell lines (Huang et al. 1995; Yokouchi et al. 1997). Thus, different members of the SH2-B family and different isoforms of SH2-B itself may be expressed differentially in distinct tissues.

SH2-B, APS, and Lnk proteins have been demonstrated to share characteristics with a number of adapter proteins, including the ability to interact with phosphorylated receptors and kinases as well as downstream signaling molecules (Huang et al. 1995; Osborne et al. 1995; Riedel et al. 1997; Wang and Riedel 1998; Yokouchi et al. 1997). Differential splicing may result in the alteration of specific motifs in SH2-B isoforms that could contribute to their function as adapter molecules. In particular, the unique C-terminal regions of SH2-Bα and SH2-Bβ contain tyrosine residues that are predicted to be sites of phosphorylation. Indeed, the tyrosine phosphorylation sites in APS and Lnk have been demonstrated to be in the C-terminal region and are homologous to the C-terminal tyrosine residue of SH2-Bα (Huang et al. 1995; Yokouchi et al. 1997). In contrast, SH2-Bγ does not have a tyrosine in its unique C-terminus.

Interestingly, the C-terminal tyrosine of SH2-Bα lies within an N-X-X-Y motif closely related to the N-P-X-Y motif that, when phosphorylated, is bound by phosphotyrosine-binding (PTB) domain signaling proteins such as Shc, members of the insulin receptor substrate (IRS) family, $p62^{dok}$ and others (Gustafson et al. 1995; Eck et al. 1996; Nelms et al. 1998; Trub et al. 1995; Zhou et al. 1996). Moreover, the C-terminal sequence of SH2-Bα, S-F-V, is an exact recognition motif for PDZ domains that have been described in a number of regulatory and signaling molecules (Harrison 1996). These putative PTB and PDZ recognition motifs may represent sites of signaling molecule interaction unique to the SH2-Bα protein. Although the C-terminal regions of SH2-Bβ and SH2-Bγ do not contain recognizable motifs, it is possible that the unique sequences in these regions are also critical to their function, perhaps by contributing to SH2 domain binding specificity or affinity.

We have also presented evidence that differential splicing of the SH2-B gene may alter the binding specificity of the encoded proteins and thus may be important in determining the signaling pathways regulated by these proteins. In particular, we have demonstrated that the binding specificity of SH2-Bγ for the IR differs from that reported for SH2-Bα (Wang and Riedel 1998). Similar to SH2-Bα, SH2-Bγ binding to the IR requires receptor phosphorylation since elimination of kinase function blocked SH2-Bγ binding (Fig. 3A). However, the interaction of SH2-Bγ with the IR requires Y1146 in the kinase domain, while SH2-Bα was reported to interact with the juxtamembrane Y960 and C-terminal Y1322 residues (Fig. 3; Wang and Riedel 1998). Our yeast two-hybrid and in vitro interaction studies clearly show that these juxtamembrane and C-terminal tyrosines are not essential for SH2-Bγ interaction. Indeed, SH2-Bγ and SHP-2 are the only signaling molecules shown to bind to phosphorylated Y1146 in the triple tyrosine motif of the IR kinase domain (Kharitonenkov et al. 1995).

The difference in SH2-Bα and SH2-Bγ binding specificity was somewhat surprising since the core of the SH2 domain is identical between these proteins. However, differential splicing results in the divergence of the C-terminal region immediately adjacent to the SH2 domain of the SH2-Bα and SH2-Bγ proteins. Thus, it is possible that these C-terminal regions contribute to phosphoprotein binding specificity. It will be of interest to determine how the unique C-terminal sequences of SH2-Bβ affect its binding to the IR and whether the SH2-Bα and SH2-Bγ isoforms bind to Jak2 and related kinases.

Together, these results suggest that differential splicing can alter the expression of functional motifs in the C-termini of SH2-B protein isoforms and may affect SH2 domain binding specificity. Indeed, control of this differential splicing may represent a unique mode of SH2-B protein regulation. In this regard, it is interesting to note that alternatively spliced forms of another IR-interacting molecule, GRB-IR, retain or delete a functional PH domain and thus have been proposed to play distinct roles in IR signaling (O'Neill et al. 1996).

The SH2-B gene maps to the distal arm of mouse Chr 7 (Fig. 4). The high degree of homology between the SH2-B proteins, APS, and Lnk suggests that the genes encoding these proteins evolved from gene duplication. In this regard, it is possible that the genes encoding APS and Lnk may also be found in this region of Chr 7, since genes encoding related signaling molecules can be genetically linked (Copeland et al. 1995). Indeed, APS has been preliminarily mapped to Chr 7 (T.A. Gustafson, unpublished data). It also will be important to determine whether alternate splicing results in the expression of different isoforms of the APS and Lnk proteins.

The specific role played by different SH2-B proteins in different signaling pathways has yet to be fully elucidated. Clearly these proteins and the related APS and Lnk proteins have the characteristics of adapter molecules. However, it is also possible that the specific interaction of SH2-Bγ with phosphorylated Y1146 in the IR kinase domain reflects a role for this protein in the regulation of kinase function. It may be significant that the SH2-B gene was localized to a region of mouse Chr 7 that has been proposed to contain a gene or genes involved in the regulation of fat metabolism in a multifactorial model of obesity in mice (Warden et al. 1993, 1995). Additional candidate genes such as *tubby* and the mitochondrial uncoupling protein 2 have also been mapped to this region (Fig. 4B; Kleyn et al. 1996; Fleury et al. 1997). However, it is tempting to speculate that mutations leading to alterations in the expression, function, or splicing of the SH2-B gene could affect insulin signaling in vivo and lead to changes in fat metabolism.

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