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Identification of Novel Alternatively Spliced Transcripts of *RBMS3* in Skeletal Muscle with Correlations to Insulin Action *in vivo*

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Whole-body insulin resistance results largely from impaired insulin-stimulated glucose disposal in skeletal muscle. Our previous studies using differential display and quantitative real-time RT-PCR have shown that a novel cDNA band (DD23) had a higher level of expression in insulin resistant skeletal muscle and it was correlated with whole-body insulin action, independent of age, sex, and percent body fat. In this study, we cloned and characterized DD23. The DD23 sequence is part of the 3' UTR region of the RNA binding motif, single stranded interacting protein (RBMS3). We have cloned the full length cDNA for *RBMS3* and identified two splice variants. These variants named DD23-L and DD23-S have 15 and 14 exons respectively and differ from *RBMS3* in the 3' UTR significantly. Northern blot analyses showed that an ~8.8 kb mRNA transcript of DD23 was predominantly expressed in skeletal muscle and to a lesser extent in placenta, but not in heart, brain, lung, liver, or kidney, unlike *RBMS3*. Elevated expression levels of these novel alternatively spliced variants of *RBMS3* in skeletal muscle may play a role in whole body insulin resistance.

Key Words: Pima Indian, Alternative splicing, c-myc, MSSP, RACE, RBMS3, Skeletal muscle, Insulin resistance

INTRODUCTION

RBMS3 is a member of the c-myc gene single-strand binding protein (MSSP) family. The MSSP family has been shown to regulate the *myc/ras* cooperative transforming activity (Niki et al., 2000b), apoptosis (Iida et al., 1997), DNA replication (Niki et al., 2000a; Takai et al., 1994), transcription (Kimura et al., 1998), and cytoplasmic RNA metabolism (Penkov et al., 2000). In addition to *RBMS3*, the MSSP family includes *RBMS1* and *RBMS2* genes (Penkov et al., 2000). *RBMS1* gives rise to alternatively spliced transcripts encoding YC1, scr2, MSSP-1, MSSP-2, and MSSP-3 (Haigermoser et al., 1996; Penkov et al., 2000). *RBMS2* and *RBMS3* encode scr3 and RBMS3, respectively (Penkov et al., 2000).

Whole-body insulin resistance results largely from impaired insulin-stimulated glucose disposal in skeletal muscle (DeFronzo et al., 1992), and is characteristic of people with type 2 diabetes mellitus (T2DM) (Bogardus, 1996). We conducted this study in Arizona Pima Indians, a population with a high prevalence of T2DM (Knowler et al., 1978), and in whom insulin resistance predicts the development of T2DM independent of obesity (Lillioja et al., 1993). Although there is scant evidence in literature, of the role of MSSPs/RBMS3 in insulin action or resistance, we report the identification of novel alternatively spliced transcripts of *RBMS3* whose skeletal muscle mRNA expression concentration had been shown to correlate with

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insulin action to mediate glucose disposal *in vivo* (Lee et al., 2003).

MATERIALS AND METHODS

Subjects and RNA isolation

This study was approved by the Tribal Council of the Gila River Indian Community and the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases. All subjects had provided written informed consent prior to their participation.

Percutaneous needle biopsies were performed on the vastus lateralis muscle under local anesthesia with 1% lidocaine (Majer et al., 1996) and the biopsy specimens were immediately frozen in liquid nitrogen. For the 5'- and 3'-rapid amplification of cDNA ends (RACE), total RNA was isolated from the frozen tissues homogenized in TRIzol Reagent (Life Technologies, Gaithersburg, MA, USA).

Northern blot analysis

Tissue distribution of DD23 was determined by Northern blot analysis using a commercial filter containing 2 μg of human poly-A RNA from adult heart, brain (whole), placenta, lung, liver, skeletal muscle, kidney, and pancreas tissues in each lane (Human Multiple Tissue Northern, Clontech, Palo Alto, CA, USA). Hybridization and washes were performed according to the manufacturer's instructions using a ^{32}P-labelled 280 bp probe within the 1.46 kb sequence of the DD23 cDNA band. Blot was prehybridized in ExpressHyb (Clontech) for 30 min at 68℃ and hybridized for 1 h at 60℃. After several washes with wash solution 1 (2X SSC, 0.05% SDS) and wash solution 2 (0.1X SSC, 0.1% SDS), the membrane was exposed to Biomax MR film (Kodak, Rochester, NY, USA) at -70℃.

Isolation of a full-length cDNA of DD23

To amplify the full length of DD23, 5'- and 3'-rapid amplification of cDNA ends (RACE) were preformed using total RNA with SMART RACE cDNA Amplification Kit (Clontech). RACE PCR products were run on 1% gel and isolated, cloned using TOPO XL PCR Cloning kit (Invitrogen, Carlsbad, CA, USA), and sequenced. From 5'-RACE PCR, a cDNA product was obtained with DD23 specific primers GSP1 (5'- GGG TGA ACT CTT GTC TCT CAC ATC -3') and GSP2 (5'- GAT TGA AGT GAG AGG CTG TCA GTC -3'). Primer GSP3 (5'- GCT GAA GTA GAG GTG CAG TCA AGC -3') was used for 3'-RACE. The second 5' RACE PCR was performed using the first RACE product specific primers, GSP4 (5'- CAT AAT GGT GAG TGT GAC CAG TTG G -3') and GSP5 (5'- TAT GGA GTC ATT CTC TGA TTT GG -3'), since the 4.5 kb PCR product of the first 5'-RACE did not contain an entire open reading frame.

RESULTS

Identification of DD23

We isolated 2 full length cDNAs for DD23 using 5'- and 3'- RACE. Sequencing of the two transcripts (DD23-L and DD23-S) showed that the DD23 (1.46 kb) is a part of the 3' UTR region in both. Each transcript consisted of a 3' UTR of ~6.5 kb terminated by a string of A residues and open reading frames of 1,314 bp and 1,260 bp encoding 437 and 419 amino acids, respectively (Fig. 1 and 2). Their sequences were novel and have been submitted to the GenBank (accession numbers: AY236871 for DD23-L and AY236872 for DD23-S). Genome BLAST searches revealed that DD23-L was encoded by 15 exons spanning 710 kb of genomic sequence (Fig. 1). DD23-S transcript was encoded by only 14 of the 15 exons (Fig. 1B) and exon 2 varied from DD23-L by deletion of 3 bases. These two transcripts appeared to be splice variants of a gene encoding the RNA binding motif, single stranded interacting protein (RBMS3) which is located on chromosome 3p24-p23 and is composed of 15 exons that span about 710 kb (Penkov et al., 2000) (Fig. 1). However exon 1 of *RBMS3* differs from those of its splice variants due to a one base (G) deletion polymorphism located 25 bp upstream from starting codon in *RBMS3* transcript. In the *RBMS3* transcript, exon 14a is continuously extended from exon 14 with an in-frame stop codon. It is noteworthy that the sequences of the 3' UTR regions of DD23-L and DD23-S are completely different from that of the previously reported *RBMS3* transcript (Fig. 1).

Since RBMS3 is a member of the *c-myc* gene single-strand binding protein (MSSP) family, the ORFs of DD23-L and DD23-S were compared with all known MSSPs (RBMS3, MSSP-1, MSSP-2, MSSP-3, YC1, scr2, and scr3) in Fig. 2 (Haigermoser et al., 1996; Negishi et al., 1994; Penkov et al., 2000; Takai et al., 1994). Similar to the other MSSPs, the predicted amino acid sequences of DD23-L and DD23-S contained two sets of ribonucleoprotein consensus sequences (RNP-CS), which are required for DNA binding (Takai et al., 1994). Furthermore, hydropathy analysis according to the Kyte-Doolittle method (Kyte and Doolittle, 1982) revealed that the predicted peptides were highly hydrophilic without a transmembrane domain (Fig. 3).

Tissue Distribution of DD23

The result of Northern blotting analysis using human multiple tissue Northern (MTN™) blot indicated that the DD23 corresponding transcript was approximately 8.8 kb and predominantly expressed in skeletal muscle and slightly in placenta but not in heart, brain, lung, liver, or kidney (Fig. 4).

DISCUSSION

Our previous differential display PCR study revealed that DD23, a novel cDNA band with higher skeletal muscle expression in insulin resistant Pima Indians, negatively correlated with glucose disposal rates at physiological insulin concentrations during a hyperinsulinemic-euglycemic clamp, and positively with fasting plasma insulin concentration, independent of age, sex, and percent body fat (Lee et al., 2003). In the current study, we have identified DD23 as a

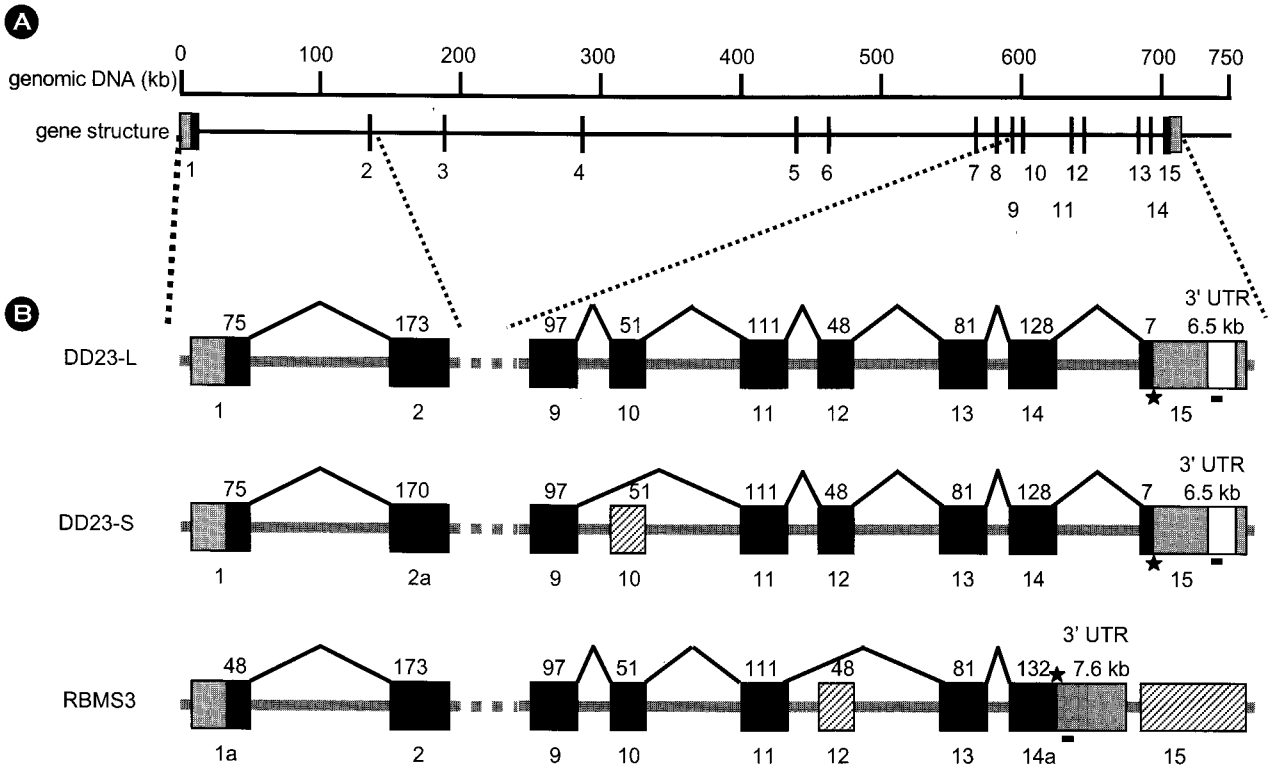

Fig. 1. Genomic organization of *RBMS3* gene and splicing events of DD23-L and DD23-S compared to RBMS3. **(A)** Schematic representation of the genomic structure of *RBMS3* gene. Exons with their corresponding numbers below are shown as black vertical bars and introns as horizontal lines. **(B)** Regions comprising exons 1 and 2 and 9~15 are magnified. Boxes represent the exons with their corresponding numbers below. Hatched boxes represent missing exons and differences between the transcripts. Numbers above the exon boxes indicate sizes of coding sequences in bp and 3'-UTR in kb. In-frame stop codons are marked with asterisks. The open boxes in 3'-UTR show the region of the differentially expressed cDNA fragment (DD23; 1460 bp) in the previous DD-PCR experiment (Lee et al., 2003), and the black horizontal bars below the 3'-UTR of DD23-L and DD23-S indicate the probe used for the Northern analyses in this study. In RBMS3 transcript, the horizontal bar below the 3'-UTR of RBMS3 indicates the site of the probe which was used in Northern blots, to study the tissue distribution of RBMS3 (Penkov et al., 2000).

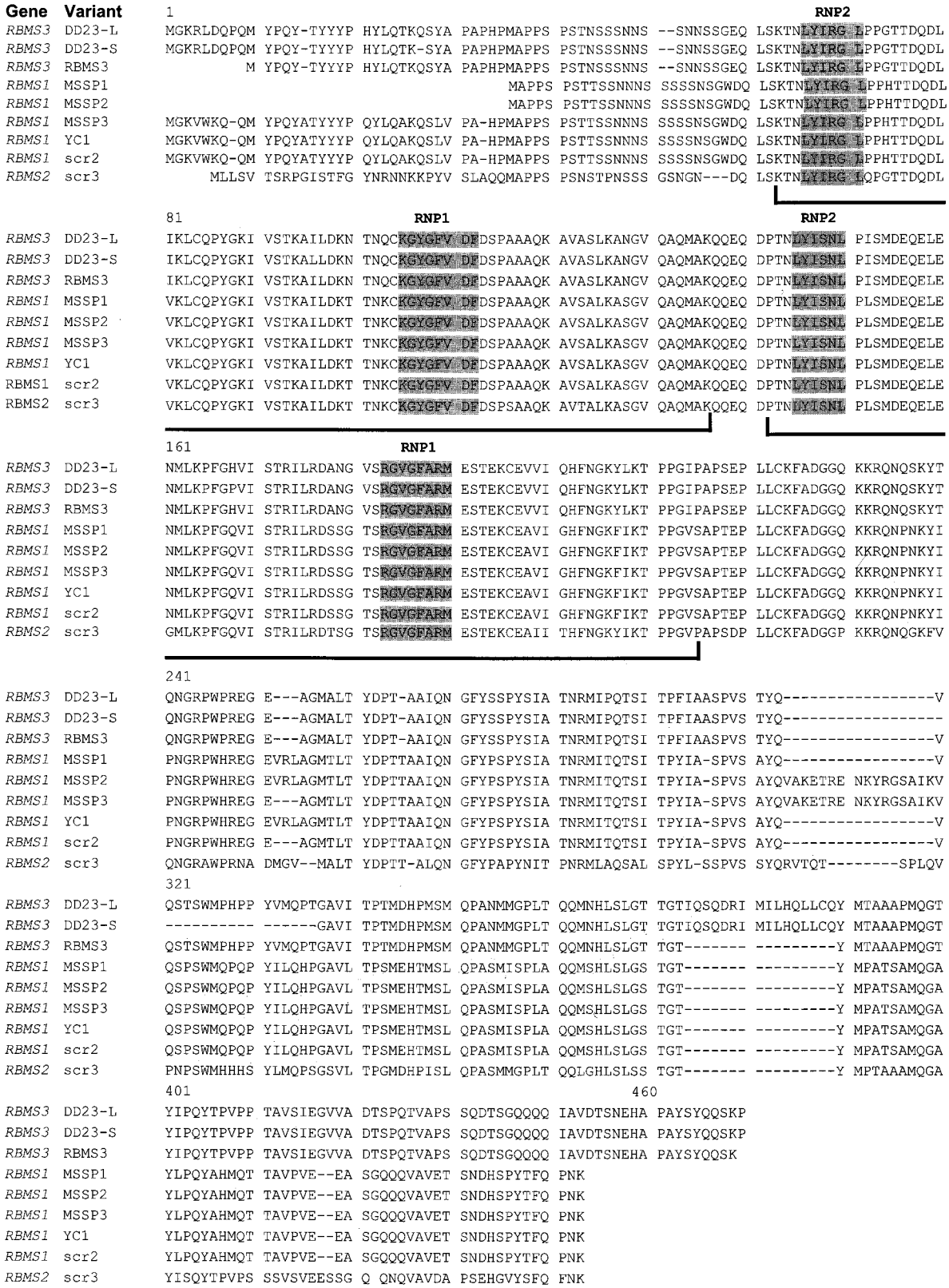

Fig. 2. Alignment of the predicted amino acid sequences of the family of MSSP proteins. Amino acid sequences were predicted from GeneBank accession nos. NM_002897 (scr2), NM_016839 (MSSP2), NM_016838 (MSSP1), NM_016837 (MSSP3), NM_016836 (YC1), NM_014483(RBMS3), NM_002898 (RBMS2). The ribonucleoprotein consensus sequence (RNP-CS) motifs and two sets of the short sequences of RNP peptides are underlined and shaded, respectively (Dreyfuss et al., 1993; Penkov et al., 2000).

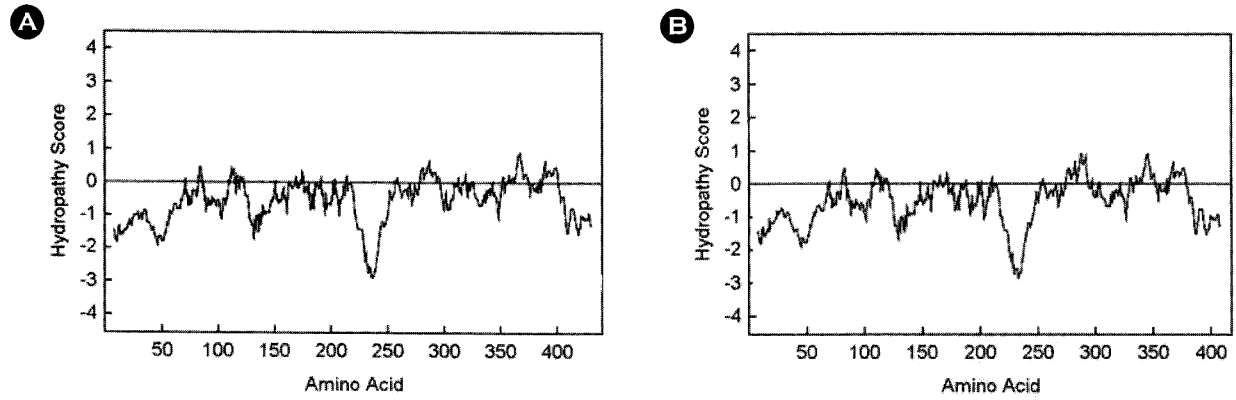

Fig. 3. Hydropathy plots of the deduced sequences of DD23-L **(A)** and DD23-S **(B)**. Hydropathy profiles were generated using the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982) with a window of nineteen residues. Positive and negative values represent hydrophobic and hydrophilic regions, respectively.

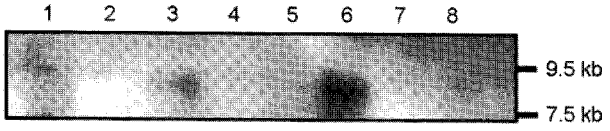

Fig. 4. Tissue distribution of DD23 by Northern blot analysis. Each lane includes about 2 μg of poly-A RNA from eight different human tissues; heart (1), brain (2), placenta (3), lung (4), liver (5), skeletal muscle (6), kidney (7), and pancreas (8).

part of the 3' UTR region of two splice variants (DD23-L and DD23-S) of a gene encoding RNA binding motif, single stranded interacting protein (*RBMS3*). *RBMS3* is located on chromosome 3p24-p23, a region which has suggestive linkages to fasting plasma insulin concentrations in non-diabetic Pima Indians (Pratley et al., 1998), and fasting plasma C-peptide/glucose ratio and fasting plasma C-peptide concentration of diabetic subjects in the Finland-United States Investigation of NIDDM Genetics (FUSION) study (Watanabe et al., 2000).

Northern blot analysis of various human tissues indicated that DD23 corresponding mRNAs (DD23-L and DD23-S) were predominantly expressed in skeletal muscle and slightly in placenta but not in heart, brain, lung, liver, and kidney, while the *RBMS3* mRNA was widely expressed in the embryo and adult tissues (Penkov et al., 2000). The predominant presence of DD23 corresponding mRNAs in skeletal muscle supports a potential role in regulating whole body insulin sensitivity. The tissue distribution and mRNA transcript levels of DD23 corresponding mRNAs as assessed by Northern analyses and quantitative real-time RT-PCR (Lee et al., 2003), respectively, is specific for these splice variants since DD23 sequence is not part of the 3' UTR of the *RBMS3* transcript.

MSSPs bind to a 21 bp sequence located ~2 kb upstream from the human c-myc gene which was identified as a putative DNA replication origin and a transcriptional enhancer (Iguchi-Ariga et al., 1988). MSSPs also bind to the C-terminal portion of c-myc protein (Niki et al., 2000b). The binding function of MSSPs is mediated through two sets of ribonucleoprotein consensus sequences (RNP-CS) (Dreyfuss et al., 1993; Takai et al., 1994). Each of these two domains contains two short sequence motifs, RNP1 and RNP2, which are highly conserved among RNA-binding proteins (Dreyfuss et al., 1993). Based on the presence of ribonucleoprotein consensus sequences (RNP-CS) for DNA binding (Takai et al., 1994) in the predicted peptides encoded by DD23-S and DD23-L, their binding properties are most probably similar to RBMS3 and other known MSSPs.

Hydropathy analysis revealed that predicted peptides encoded by DD23-L and DD23-S were highly hydrophilic without a transmembrane domain. This indicates that the predicted peptides most likely reside in cytoplasm, similar to the localization of RBMS3 (Penkov et al., 2000), and/or nucleus, as implicated by the DNA binding function of other MSSPs. The cytoplasmic localization and RNA binding properties of RBMS3 indicate its involvement in cytoplasmic RNA metabolism (Penkov et al., 2000). Thus, DD23-S and DD23-L encode peptides that may also regulate RNA metabolism and/or bind to c-Myc or other target

proteins via the RNP motif to regulate diverse cellular functions.

So far, since there has not been any report of direct association between RBMS3 or MSSPs with insulin action. Therefore we do not know if the increased mRNA expression of DD23-L and DD-23-S contribute to or is a consequence of insulin resistance. However, differences in expression levels of the c-myc gene have been implicated in insulin resistance in various glucose responsive tissues (Thompson et al., 1996; Weir et al., 2001). Since RBMS3/MSSPs/DD23 L and DD23-S do have c-myc binding motifs, they may cooperate with c-myc protein to regulate DNA replication, gene transcription, induction of apoptosis and cell-cycle progression. One can hypothesize that together, they may play a role in cellular control of genes more directly involved in insulin resistance such as the lactate dehydrogenase gene (LDH-A) (Dang, 1999) whose product participates in normal anaerobic glycolysis (Osthus et al., 2000; Shim et al., 1997; Tavtigian et al., 1994). Nevertheless, the exact relationship between DD23-L and DD23-S and c-Myc or other proteins involved in insulin action needs to be clarified by further experiments.

In summary, we cloned, identified and preliminarily characterized a novel cDNA band, DD23, which showed increased expression in insulin resistant skeletal muscle and correlated with whole-body insulin action. The cDNA band corresponds to DD23-L and DD23-S, two alternatively spliced variants of *RBMS3*, a member of MSSP family. They are localized predominantly in skeletal muscle, supporting their role in muscle insulin sensitivity.

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