Characterization of Two Distinct Dual Specificity Phosphatases Encoded in Alternative Open Reading Frames of a Single Gene Located on Human Chromosome 10q22.2*

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Dual specificity phosphatases (DSPs) are members of the protein-tyrosine phosphatase superfamily that dephosphorylate both phosphotyrosine and phosphoserine/threonine residues in vitro. Many DSPs have been found to play important roles in various aspects of cellular function and to be involved in human disease. We have identified a gene located on human chromosome 10q22.2, which utilizes alternative open reading frames (ORFs) to encode the following two distinct DSPs: the previously described testis and skeletal muscle-specific dual specificity phosphatase (TMDP) and a novel DSP, muscle-restricted dual specificity phosphatase (MDSP). Use of alternative ORFs encoding distinct proteins from a single gene is extremely rare in eukaryotes, and in all previously reported cases the two proteins produced from one gene are unrelated. To our knowledge this is the first example of a gene from which two distinct proteins of the same family are expressed using alternative ORFs. Here we provide evidence that both MDSP and TMDP proteins are expressed in vivo and are restricted to specific tissues, skeletal muscle and testis, respectively. Most interestingly, the protein expression profiles of both MDSP and TMDP during mouse postnatal development are strikingly different. MDSP is expressed at very low levels in myotubes and early postnatal muscle. TMDP is not detectable in testis lysate in the first 3 weeks of life. The expression of both MDSP and TMDP proteins was markedly increased at approximately the 3rd week after birth and continued to increase gradually into adulthood, implying that the physiological functions of both DSPs are specific to the mature/late-developing organs. The conserved gene structure and the similarity in postnatal expression profile of these two proteins suggest biological significance of the unusual gene arrangement.

Members of the protein-tyrosine phosphatase (PTP)1 superfamily are characterized by the presence of a signature motif (HCX_R) in their primary sequences. With the completion of the genome sequencing projects, we are now aware of ~100 PTP genes in the mammalian genomes. The PTP superfamily is roughly divided into two categories according to substrate specificity, the tyrosine-specific phosphatases and the dual specificity phosphatases (DSPs). The DSPs are operationally defined as members of the PTP superfamily that have the capability to dephosphorylate both phosphotyrosine and phosphoserine/threonine residues in vitro. Nevertheless, in terms of physiological function, the “DSPs” may actually show preference for either Ser/Thr (1, 2) or Tyr residues (3). Most interestingly, some DSPs even target nonprotein substrates such as phospholipids (4, 5) and RNA (6) in vivo. Although members of this family have been shown to play pivotal roles in various aspects of cell function, including signal transduction, actin polymerization, mRNA processing, cell cycle regulation, and apoptosis (7–9), the physiological functions of many DSPs remain to be elucidated.

The prototypic DSP is VH1, a virulence factor of the vaccinia virus (10, 11). This open reading frame encodes a monomeric catalytic subunit with a molecular weight of ~20,000 (12). Now it is known that the DSPs are a heterogeneous group that includes the VH1-like enzymes, the Cdc25-related phosphatases, and the myotubulin-related phosphatases (MTMs). Cdc25s are important regulators of cell cycle progression (reviewed in Refs. 13 and 14), and the MTMs are phosphoinositide phosphatases implicated in human diseases (reviewed in Refs. 15 and 16). The VH1-like DSPs are an ancient group of enzymes found in archaeobacteria, cyanobacteria, viruses, yeasts, plants, and animals. We have identified 43 VH1-like DSPs in the human genome, all but one of which are also found in mouse.2 Many VH1-like DSPs have been shown to function as negative regulators of MAPK signaling pathways. MAPKs are activated by dual phosphorylation on both the Thr and the Tyr residues in the TXY motif of their activation loop, catalyzed by kinases upstream in the signaling pathway (17, 18). Dephosphorylation of these residues returns the MAPKs to their inactive state. This can be achieved either by single specificity PTPs and protein Ser/Thr phosphatases acting on the individual Tyr and Thr phosphorylation sites (reviewed in Refs. 19

1 The abbreviations used are: PTP, protein-tyrosine phosphatase; DSP, dual specificity phosphatase; TMDP, testis- and skeletal muscle-

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specific dual specificity phosphatase; MDSP, muscle-restricted dual specificity phosphatase; MAP, mitogen-activated protein; MAPK, MAP kinase; MKP, MAP kinase phosphatase; VHR, VH1-related protein-tyrosine phosphatase; ORF, open reading frame; RT-PCR, reverse transcription-PCR; HA, hemagglutinin; GST, glutathione S-transferase; MBP, maltose-binding protein; RCML, reduced carboxyamidomethylated and maleylated lysozyme; DYRK, dual specificity tyrosine-regulated kinase; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; WT, wild type; Erk, extracellular signal-regulated kinase; Jnk, c-Jun NH2-terminal kinase; h, human; m, mouse.

2 H.-H. Chen et al., manuscript in preparation.
and 20) or by DSPs, termed the MKPs (MAP kinase phosphatases), which dephosphorylate both residues (reviewed in Refs. 21–25). Ten mammalian MKPs contain an additional sub-strate-binding domain N-terminal to their VH1-like DSP catalytic domain, which confers specificity upon the interaction between the MKP and specific MAP kinases (26–29). In addition, several other small VH1-like DSPs, which lack the N-terminal regulatory motif, are also reported to target MAPKs as physiological substrates, and these include VHR and MKP6 (3, 30–32).

VH1-related (VHR) protein-tyrosine phosphatase, the first mammalian VH1-like DSP to be identified (33), is a single domain protein of 20 kDa and is expressed in most cell lines and tissues (3, 30). VHR has been shown to target Erk, Jnk, and p38 as substrates, preferentially dephosphorylating the phosphoryosyl residue in the TXY motifs of MAPK (3, 34).

Structural studies on VHR illustrate that the amino acids contributing to this preference for tyrosine include two unique bulky residues in its signature motif (35). Four other VH1-like DSPs in the human genome were found to be closely related to VHR; two are gestis and skeletal muscle-specific dual specificity phosphatase (TMDP) (36) and three novel DSPs. The mRNA of TMDP is detected only in skeletal muscle and testis, and based on mRNA localization and expression in development, TMDP is proposed to be involved in spermatogenesis (36). However, whether TMDP protein is expressed in these two tissues was not suggested.

Here we describe the characterization of a gene (DUSP13 locus) encoding two distinct VH1-like DSPs. One product from this gene is one of the three novel DSPs that are also closely related to VHR, and the other is the previously characterized TMDP. These two DSPs share no overlapping coding sequence. We have detected the endogenous expression of both DSP proteins, and we characterized the mRNA species produced from this gene, proving that two closely related phosphatases are generated from different open reading frames (ORFs) in one gene. Both proteins showed strict, yet distinct, tissue specificity. Our data also implied that both DSPs are involved in postnatal development of specific tissues. Based on the specific tissue expression patterns, we named the novel DSP MDSP (muscle-restricted dual specificity phosphatase). To our knowledge, the DUSP13 locus is the first gene from which two distinct proteins of the same family are expressed using alternative ORFs.

**Experimental Procedures**

**Northern Blot Analysis and RT-PCR Analysis—**Polyadenylated RNAs from human skeletal muscle and testis were purchased from Clontech. Primers used for RT-PCR and generation of templates by PCR for Northern blotting are as follows: Ex1F (5'-ATGGCTGA-AGACCTCTTCTGCAGCA-3') and Ex1R (5'-GCATCTCTTCTGTTAGGCA-3'); Ex2F (5'-GCCACCCGCAAACAGCTGTTTG-3') and Ex2R (5'-CAGGCGGTTTAGGGCCAGCTGGA-3'); Ex3F (5'-CC-AAGGTCTGTCCTGACTGT) and Ex3R (5'-TCAGCTGTTCGCCGCACGG-3'); Ex4F (5'-ATGGGCTCTGCTCCAGGCACGAG-3') and Ex4R (5'-GTATCTGCCCAGGCTGCCTTCTC-3'); and Ex7F (5'-AGTCACGGAGACAGGTCTGTTG-3') and Ex7R (5'-TCAGACCCGGCTCTGCTC-3'). Primer pairs used to generate probe templates are as follows: exon 1, Ex1F and Ex1R; exon 2, Ex2F and Ex2R; exon3, Ex3F and Ex3R; exon4-5, Ex4F and Ex5R; exons7,8, Ex7F and Ex9R. Primer pairs for RT-PCR are as follows: A, Ex7F and Ex9R; B, Ex4F and Ex9R; C, Ex1F and Ex3R; and D, Ex1F and Ex9R. RT-PCR was performed with Superscript One-Step RT-PCR (Invitrogen). The sense primers for GAP-32PdCTP/PerkinElmer Life Sciences and with the ProbeQuant G-50 Micro Columns (Amersham Biosciences).
was included in buffers as indicated in the figure legends. For immunoprecipitation of overexpressed HA-tagged MAP kinases, 2 μl of monoclonal antibody 12CA5 reacts bound to 10 μl of protein A-Sepharose and 0.5–1.5 mg of protein lysate were used in each reaction. Mouse and rat tissues were homogenized in standard RIPA buffer or Lysis Buffer using a hand-held Dounce homogenizer. For immunoprecipitation of endogenous TMDD, purified polyclonal antibodies CS1911 (40 μg) or CS1912 (20 μg) bound to 40 μl of protein A-Sepharose and 8 μg of rat testis lysate in RIPA buffer were used. Concentrations of purified antibodies used for immunoblotting of MDSP and TMDD were as follows: CS1911 (3 μg/ml), CS1912 (2 μg/ml), CS1914 (1 μg/ml), CS1875 (4 μg/ml), and CS1500 (10 μg/ml). Commercial antibodies were purchased from the following sources: monoclonal anti-rat tubulin clone B-5-1-2, Sigma; PY20, Transduction Laboratories.

Phosphatase and Kinase Assays—Activity toward p-nitrophenyl phosphate was measured in 96-well microplates at 25 °C in a 100-μl incubation mixture containing a three-component buffer (38) (100 mm acetic acid, 50 mM Bistris, and 50 mM Tris, pH 6.0, or 7.0, with 2 mM diithiothreitol) at increasing concentrations of p-nitrophenyl phosphate from 0 to 20 μM, using 0.5–4.0 μg of GST-hMDSP in each reaction. Reactions were terminated at 20 min or as indicated with 100 μM of 0.1 M NaOH, 50% ethanol. The absorbance at 410 nm was measured using a microplate reader. The concentration of p-nitrophenolate produced was calculated using a molar absorptivity of 1.78 × 10^4 M⁻¹.

Reduced carboxyamidomethylated and maleylated lysozyme (RCML) and phosphorylated synthetic substrates (poly(Glu-Tyr) (Sigma) were phosphorylated in the presence of γ-32P]-ATP with β-IRK or PPA as described in Ref. 39. Phosphatase assays using proteinaceous substrates were performed at 30 °C in a 60-μl mixture containing 25 mM imidazole, pH 7.2, 10 mM diithiothreitol, 0.1 mg/ml bovine serum albumin, 5 μM phosphorylated residues on substrates using 0.7–8 μg/ml of GST-hMDSP.

In-gel phosphatase assays of the endogenous TMDD from rat testis were performed as described in Ref. 40 with the following modification. Because endogenous TMDD has the same apparent molecular weight as the rabbit immunoglobulin light chain, SDS-PAGE for in-gel assay and the immunoblotting were conducted under nonreducing conditions (no diithiothreitol) without iodoacetic acid. MAP kinase assays were conducted as described in Ref. 41.

Cell Culture and Transfection—COS1 cells and C2C12 myoblasts were maintained at 37 °C, 5% CO_2 in Growth Media (10% fetal bovine serum, 1% penicillin/streptomycin in Dulbecco's modified Eagle's medium). Transfections were conducted using various expression constructs and the FuGENE 6 reagent (Roche Applied Science). Cells are generally harvested 36–72 h post-transfection. Primary myoblasts were isolated from 4- to 6-week-old mouse limbs by mincing and protease digestion followed by centrifugation in a 40–70% Percoll gradient. Myoblasts were maintained at 37 °C, 5% CO_2 in 20% fetal bovine serum, 5 ng/ml FGF-2, 1% penicillin/streptomycin in F-10 media. Myocyte formation of both C2C12 cells and primary myoblasts was induced with differentiation media (2% horse serum, 1% penicillin/streptomycin in Dulbecco's modified Eagle's medium).

Indirect Immunofluorescence—C2C12 cells were seeded on acid-washed coverslips and incubated in Growth Media. Cells were switched into Differentiation Media after reaching ~90% confluence. Differentiated myotubes were transfected on the 4th day in Differentiation Media and fixed in 4% paraformaldehyde on the 7th day. Undifferentiated C2C12 myoblasts, COS1 cells, and HeLa cells were fixed on the 3rd day after transfection.

RESULTS

Two DSPs Were Encoded in Alternative ORFs from One Gene—In our search for novel DSPs in the human genome, we found sequences encoding two distinct DSP catalytic domains located close to each other on human chromosome 10q22.2. Each of the ORFs spanned nonoverlapping exons (Fig. 1A). One of the DSPs is the human form of TMDD, a murine phosphatase referred to as Testa and Muscle-specific Dual specificity Phosphatase (P48). The second was a novel protein, which we have named MDSP, Muscle-restricted Dual Specificity Phosphatase (Fig. 1B, GenBank™ accession numbers AY674051 (human MDSP) and AY674052 (mouse MDSP)). We have conducted a phylogenetic analysis of the VH1-like DSPs found in the human genome and observed that MDSP and TMDD are closely related (42% identity in amino acid sequence). They belong to a subgroup of DSPs containing five mammalian proteins, of which the best characterized is VHR (38% identity with MDSP and 37% identity with TMDD) (33).

Comparison of this gene to the corresponding locus in the mouse genome showed that the gene structure is well conserved between human and mouse (Fig. 1C), and the murine locus would be predicted to encode proteins of 85 and 87% identity to human MDSP and TMDD, respectively. Both the size and the nucleotide sequence identity are conserved in the 9 exons identified from available EST sequences, whereas the majority of the intronic sequences are not conserved. In the genome of Fugu rubripes, the VHR-like subgroup of DSPs appeared to be expanded, whereas the majority of the other VH1-like DSPs in Fugu showed one-to-one matches to the human DSPs. At least 13 VH1-like DSP sequences were found in the Fugu genome, with multiple pairs located closely on the genomic sequence scaffolds. The arrangement of the genomic sequence of one of those pairs was similar to that of the mammalian DUSP13 locus, suggesting that this gene structure may also be conserved in Fugu.

Detection of MDSP and TMDD Transcripts in Specific Tissues—The conservation of the gene structure between species suggested functional significance. Therefore, we investigated whether each of the two ORFs were transcribed into messenger RNA. Probes against the TMDD ORF were used to screen a multiple human tissue Northern blot. Expression of the TMDD sequence was only detected in skeletal muscle and testis among the 16 human tissues examined (data not shown); this result is similar to the original observation for the mouse gene (38). Multiple bands of varying sizes from 1 to 1.8 kb were detected from both tissues. To decipher the different transcripts expressed in skeletal muscle and testis, we examined further the expression pattern of different exons in each of the two tissues using Northern blot and RT-PCR analyses. DNA probes were generated against exon 1, exon 2, exon 3, exons 4-5, and exons 7–9, respectively, and each of the probes was tested for specificity by Southern dot blot analyses (data not shown). The exon-specific probes were used to detect the expression of the exon in human skeletal muscle and testis mRNAs by Northern blotting. We found that mRNA species containing exons 1–3, which represent the MDSP ORF, could be detected in skeletal muscle but not in testis, whereas exons 7–9, which represent the human homologue of TMDD, were expressed at comparable levels in both tissues (Fig. 2A). In addition, exons 4 and 5 are also only expressed in skeletal muscle.

To determine whether exons 1–5 are located on the same transcripts as exons 7–9 in skeletal muscle, we performed nonquantitative RT-PCR analysis using exon-specific primers (Fig. 2B). Transcripts containing exons 7–9 were present at comparable levels in both skeletal muscle and testis, whereas mRNA containing exons 1–5 are present at much higher amounts in skeletal muscle, although trace amounts of expression can be detected in testis by RT-PCR. We found multiple PCR products at much higher levels in skeletal muscle than in testis when a forward primer specific to exon 1 and a reverse primer specific to exon 9 (primer pair D) were used. We cloned this pool of PCR products from skeletal muscle and determined the identity of these products by DNA sequencing (Fig. 2C). Out of the 75 clones we sequenced, 8 different combinations of exons were present. Three of the eight contained ORFs encoding full catalytic domains of either MDSP or TMDD (TMDD-L1 and TMDD-L2, putative longer forms of TMDD in muscle). Although these three transcripts were present in skeletal muscle, results from immunoblot analyses shown below indicate that only the longest transcript encoding MDSP is translated into protein product, whereas TMDDPL1/L2 proteins were not detected.
FIG. 1. The unique gene structure encoding two closely related yet distinct dual specificity phosphatases. A, schematic representation of the human gene located on chromosome 10q22.2. Two DSP catalytic domains are encoded within a region of 14.4 kb. Exons 1–3 of this gene encode the MDSP ORF, a novel phosphatase, whereas exons 7–9 encoded the TMDP ORF, illustrated by the boxes. The extended signature motif sequence for each DSP is shown above or below the boxes representing the ORFs. The skeletal muscle-specific (top) and the testis-specific (bottom) transcriptional start sites are shown as arrows. The skeletal muscle-specific exons are shown as light gray boxes, the testis-specific exon is shown as a dark gray box, and the exons expressed in both tissues are shown as white boxes. Introns are shown as horizontal black lines. Splicing events are shown as connecting lines above or below the exons. Exons and introns are not drawn to scale. B, protein and cDNA sequence of human MDSP. The DNA sequence encoding MDSP is shown, and the translation of the MDSP ORF is provided below the DNA sequence. The extended signature motif of MDSP is doubly underlined and the conserved Asp, which functions as a general acid/base in catalysis, is in boldface and boxed. The long 5'-untranslated region contains the complete TMDP ORF (underlined). C, comparison of the DUSP10 locus structure between human and mouse. A comparison of nucleotide sequences between the homologous regions in human and mouse showed that the MDSP/TMDP gene structure is conserved in evolution. The exons are shown in boxes and the introns in solid lines above and below the exon boxes (not to scale). The percentage identity in nucleotide sequence between the exons of human and mouse genes is presented.
The Northern and the RT-PCR data showed that transcripts containing exon 1 were expressed in skeletal muscle but not in testis, suggesting that a skeletal muscle-specific promoter is located upstream of exon 1. The 5'-UTR sequence of human TMDP mRNA (GenBank™ accession number AB027004, Nakamura et al. (36)) contains an additional exon, exon 6, that was not present in the transcripts produced from the promoter for exon 1. There is apparently a separate testis-specific promoter initiating transcription from exon 6. We conclude that at least two different promoters were used to express tissue-specific transcripts from this gene.

**MDSP and TMDP Were Expressed in a Tissue-specific Manner**—In order to ascertain whether both dual specificity phosphatases were expressed as proteins in vivo, we sought to detect the endogenous protein encoded by each ORF. Polyclonal antibodies specific to MDSP and TMDP were generated and purified as described under “Experimental Procedures.” These antibodies were used to screen for the presence of endogenous TMDP and MDSP in various mouse tissue lysates. Affinity-purified antibodies from three different rabbits immunized with recombinant TMDP protein all identified a band of ~27 kDa in lysates of mouse testis (Fig. 3A) and rat testis (Fig. 3B).

This protein species was not detected in other tissues, and no other bands were observed reproducibly with the different antibodies. Furthermore, this ~27-kDa protein, as immunoprecipitated from a lysate of rat testis, was active in an in-gel phosphatase assay (Fig. 3B). The failure to detect TMDP protein in skeletal muscle lysate suggests that the longer forms of TMDP predicted from transcripts found in skeletal muscle (TMDP-L1 and TMDP-L2) may not be translated in the normal adult tissues.

The MDSP ORF spans exons 1–3, with a termination codon in exon 3. To define RNA species encoding this ORF, we tested whether MDSP transcripts were present that lack the long 3'-UTR containing exons 4–5 and 7–9. Multiple tissue Northern analysis using probes specific for exon 2 (data not shown) or exon 3 (Fig. 4A) revealed a species of ~1.8 kb (corresponding to the size of the MDSP transcript containing exons 1–5 and 7–9) in skeletal muscle but not in any other tissues examined including testis and heart. This result indicates that the only transcript for MDSP also contained the complete TMDP ORF in its 3'-UTR. By using three different MDSP antibodies to screen lysates of multiple mouse tissues by immunoblotting, we detected bands migrating at ~21.5 kDa only in diaphragm and abdominal muscle lysates, both of which are composed of skeletal muscle (Fig. 4B). This band comigrated with recombinant untagged MDSP, of which the predicted molecular mass is 20.5 kDa. Our results showed that both the mRNA and the protein of MDSP were only expressed in skeletal muscle.

**Recombinant MDSP Was Active as a DSP in Vitro**—The primary sequences of both TMDP and MDSP contain the features known to be required for phosphatase activity. The

147 nucleotides upstream of the major 5' splice site. Of 75 clones we sequenced from lane D, 4 contained a shorter form of exon 8 (Fig. 2B), summary of multiple spliced mRNAs produced from the DUSP13 locus in skeletal muscle. The multiple bands in lane D of the human skeletal muscle panel, in the absence of RNase A (Fig. 2B), were TA-cloned as a pool and analyzed by DNA sequencing. 75 clones were sequenced, and 8 combinations of exon usage were detected. The numbers in the column entitled Spliced forms denote the exons present in each transcript, and the line below the numbers denotes putative ORFs. Arrows represent ORFs containing either DSP domain, and the vertical lines represent termination codons in both human and mouse sequence. Double vertical lines represent termination codon in mouse but not in human. The mRNA products X1, X2, X3, and X4 would not encode active DSPs because they lack the signature motifs. (Product X2 may encode a longer form of TMDP as judged from the human sequence; however, a termination codon in mouse exon 3 would be in-frame in this spliced form.)
Two Distinct Phosphatases Encoded in One Gene

A

B

Fig. 3. Expression of TMDP protein in testis but not in skeletal muscle. A, immunoblot (IB) analysis of TMDP in multiple mouse tissues. Tissue lysates (60 μg, 1% SDS buffer) were used in immunoblot analysis, and three affinity-purified antibodies against against TMDP all produced similar results. The tissues examined are as follows: B, brain; H, heart; K, kidney; Li, liver; Lu, lung; P, pancreas; M, skeletal muscle; T, testis. B, in-gel phosphatase assay of proteins immunoprecipitated (IP) from rat testis lysate using antibodies against TMDP. Affinity-purified polyclonal antibodies from two rabbits (#1, CS1911; #2, CS1912) were used to immunoprecipitate TMDP from 1 mg of rat testis RIPA lysate. The immunoprecipitates were analyzed in an in-gel phosphatase assay using 32P-labeled poly(Glu-Tyr) as substrate. Crude lysate was used as positive control, and a mock immunoprecipitation as a negative control. The presence of the TMDP protein in rat testis lysate and the immunoprecipitates was confirmed by immunoblotting with the same antibodies described above.

Fig. 4. Tissue expression of MDSP mRNA and protein. A, Northern blot analysis of MDSP mRNA expression in multiple human tissues. Probes specific to exon 3 of the DUSP13 locus were used to detect MDSP mRNA on the multiple tissue Northern blot (MTN™, Clontech). The blots were stripped and reprobed for β-actin. The tissues examined are as follows: H, heart; B, brain; P, placenta; Lu, lung; Li, liver; M, skeletal muscle; K, kidney; Pa, pancreas; Sp, spleen; Th, thymus; Pr, prostate; T, testis; O, ovary; SI, small intestines; Co, colon; PB, peripheral blood. B, immunoblot (IB) analysis of MDSP protein expression in multiple mouse tissues. Affinity-purified antibodies generated against MDSP from four different rabbits were used to detect endogenous MDSP expression in mouse tissue lysates. Arrow, MDSP protein detected by all four antibodies. The tissues examined are as follows: B, brain; D, diaphragm; H, heart; K, kidney; Li, liver; Lu, lung; Pa, pancreas; M, skeletal muscle; T, testis.

Trintrinsic activity of murine TMDP in vitro has been demonstrated in the previous report by Nakamura et al. (36). To confirm that the recombinant MDSP protein possessed intrinsic activity, we tested bacterially expressed GST-tagged MDSP against artificial substrates. The $K_m$ value of affinity-purified GST-MDSP toward p-nitrophenol phosphate is $\sim 7.5 \text{ mM}$ at pH 6.0, comparable with that of VHR ($K_m = 1.9 \text{ mM}$ at pH 6.0 (34)). In addition, GST-MDSP displayed phosphatase activity toward protein substrates containing either phosphotyrosine or phosphothreonine, and the activity in each case could be inhibited with 1 mM vanadate (Fig. 5A). These data illustrate that GST-MDSP possessed intrinsic dual specificity phosphatase activity in vitro.

MDSP and VHR Appeared to Be Functionally Distinct—VHR, the close relative of both TMDP and MDSP, has been reported to be a nuclear protein in cultured cells (3) and to be diffusely distributed in the cytosol of resting T cells (42). In light of the similarity in sequence among VHR, TMDP, and MDSP, we tested whether the latter showed some of the properties reported for VHR. To assess the subcellular localization of MDSP, we performed indirect immunofluorescence in cultured cells overexpressing untagged MDSP. C2C12 myotubes were transfectected with MDSP expression construct on the 4th day in Differentiation Media and then fixed and visualized 3 days after transfection. MDSP displayed a diffuse staining in the cytosol and was apparently excluded from all nuclei (Fig. 5B). The same results were obtained when the C2C12 myoblasts were transfected with MDSP prior to induction of differentiation. Similar subcellular localization patterns were observed in COS1 cells and HeLa cells overexpressing MDSP (data not shown). Therefore, we conclude that MDSP was primarily cytosolic.

Several VH1-like DSPs have been shown to target MAP kinases as physiological substrates, including VHR. We investigated whether MDSP can dephosphorylate a panel of MAP kinases. COS1 cells were cotransfected with GST-tagged MDSP, either wild type or an inactive mutant (R135M, in which the Arg in the signature motif is changed to Met) and an HA-tagged MAPK (Erk2, Jnk1, p38α, or p38β). Transfected cells were serum-starved, stimulated with either serum or UV light, and then harvested in Lysis Buffer containing 1 mM vanadate. The MAP kinases were immunoprecipitated and immunoblotted for phosphotyrosine. We included DSPs that have been reported to dephosphorylate MAPKs as controls, specifically GST-VHR (wild type and D92A) and Myc-tagged MKP1-1 (314) (wild type and C258S). We saw no difference in the level of phosphotyrosine in MAP kinases coexpressed with either GST-MDSP WT or R135M, whereas Myc-MKP-1 consistently dephosphorylated phosphotyrosine on MAPKs compared with its inactive control (Fig. 6). Expression of GST-VHR re-
duced the tyrosine phosphorylation in Jnk1 and to a lesser extent in p38α and p38β, although we did not observe VHR dephosphorylation of Erk2. Furthermore, we did not detect dephosphorylation of the activated MAP kinases by untagged MDSP (data not shown). To confirm that the overexpressed GST-MDSP retained phosphatase activity, we precipitated the MDSP (data not shown). To confirm that the overexpressed GST-MDSP WT and GST-VHR WT displayed significant phosphatase activity compared with their inactive mutants. We conclude that MDSP, unlike VHR and MKP1, does not recognize the phosphotyrosyl group on MAP kinases as substrate.

The MAPks are phosphorylated on both Thr and Tyr of a TXY motif in the activation loop of the kinase. The activity of Erk singly phosphorylated only on the Tyr has been shown to be 1–2 orders of magnitude lower than the fully phosphorylated form (29); therefore, a significant reduction of the threonine phosphorylation should be reflected in inhibition of kinase activity. To address the question of whether MDSP targets the phosphothreonyl residue in the TXY motif of MAP kinases as physiological substrates, we tested the effect of MDSP expression on MAP kinase activity. GST-MDSP expression did not affect the activity of coexpressed MAP kinases measured in vitro, whereas Myc-MKP1 reduced the kinase activities profoundly (data not shown). Our results suggest that MAP kinases are unlikely to be physiological substrates for MDSP.

**MDSP and TMDP Are Expressed in Adult Tissues**—In the previous study by Nakamura et al. (36), the expression levels of mRNAs containing the TMDP ORF were shown to be developmentally regulated in testis. By using the antibodies described above, we examined whether this change in mRNA level is developmentally regulated in testis. By using the antibodies described above, we examined whether this change in mRNA level is developmentally regulated in testis. By using the antibodies described above, we examined whether this change in mRNA level is developmentally regulated in testis.
Fig. 7. Expression of TMDP is up-regulated during testis development in mouse. Immunoblot analysis of TMDP expression in testis lysates from postnatal mice. The male mice were pooled from two litters of different parents. The lane numbers denote the ages of the mice (postnatal days (P)) at organ procurement. Arrow, TMDP; the band above the arrow is a background band not detected with other antibodies. Blots were stripped and reprobed with anti-α-tubulin antibody. IB, immunoblot.

7). Thus, the highest levels of TMDP were observed in adult tissue. We examined whether MDSP was expressed endogenously in cultured cells by immunoblotting. MDSP protein was not detectable in lysates of NIH3T3 fibroblasts or normal mouse embryonic fibroblasts but was detected in lysates of C2C12 myoblasts, C2C12 myotubes, and normal mouse myotubes (data not shown); however the MDSP levels in these cultured cells were much lower than that in the mature skeletal muscle. Endogenous expression of MDSP protein in cultured myotubes was comparable with that in early postnatal skeletal muscle (<2 weeks), which was 5–10% of the concentration in mature mouse skeletal muscle (data not shown). Using quantitative immunoblot analysis of recombinant MDSP, we estimated that MDSP constitutes ~0.001% of the total RIPA-soluble protein in mature skeletal muscle.

We examined whether the expression of MDSP protein was also developmentally regulated by immunoblotting in postnatal mouse skeletal muscle. The level of MDSP protein was low in neonatal muscle but showed a gradual yet prominent increase during postnatal development between the 2nd week and the 6th week after birth (Fig. 8A). Similar results were obtained in three different litters from two sets of parents. In two of the litters we weighed the frozen quadriceps muscles before homogenization. The increase in MDSP expression apparently correlated with the increase in the mass of the quadriceps muscle in both litters (Fig. 8B shows the result from one litter). There were no obvious differences in MDSP expression levels between sexes or among different muscles (quadriceps, gastrocnemius, soleus, extensor digitorum longus, and tibialis anterior) that could be detected by immunoblotting (data not shown).

DISCUSSION

Genes encoding alternative ORFs that produce two distinct proteins are rare in higher eukaryotes. The best characterized example is the INK4a locus (43–45). By using two alternative promoters, the INK4a locus encodes p16INK4a, an inhibitor of cyclin D-dependent kinases, and p19ARF (p14ARF in human), which binds to and inactivates the p53 inhibitor, Mdm2, and thereby promotes growth arrest and/or apoptosis. The coding sequences for these two proteins overlap, but they share no overlapping amino acid sequence due to usage of alternative reading frames. The two proteins have different biochemical properties and cellular functions and yet act in concert to inhibit cell cycle progression. The need for coordination in these events may therefore explain the unusual gene structure of the INK4a locus. In the other genes known to produce two closely related and yet distinct protein products, TMDP and MDSP, by use of nonoverlapping alternative ORFs.

TMDP was previously described as the testis and muscle-specific dual specificity phosphatase by Nakamura et al. (36). We have shown for the first time that the endogenous TMDP protein is present in testis and is active as a phosphatase in vitro, whereas the mRNA signal from skeletal muscle represents a mixture of different transcripts. Our analyses of the messenger RNA species indicated a potential for other forms of TMDP to be expressed in skeletal muscle (TMDP-L1 and TMDP-L2), although such protein products, if present, were not detectable with our antibodies. TMDP is thus most likely a testis-specific protein. The 5′-UTR sequence of human TMDP mRNA in testis has been reported (GenBankTM accession num-
MDSP, from a different, nonoverlapping ORF (exons 1 produced a detectable protein product, the novel phosphatase). Only one of the transcripts from this promoter (Fig. 1A) is present which likely initiates transcription at exon 1 (Fig. 1A). Only one of the transcripts from this promoter produced a detectable protein product, the novel phosphatase MDSP, from a different, nonoverlapping ORF (exons 1–3). The nonoverlapping ORFs of MDSP and TMDP are considered to be products of one gene not only because of their proximity on the chromosome, but because that the whole TMDP ORF is transcribed in the 3′-UTR of the only MDSP transcript detected. Although the two proteins have no overlapping coding region, their transcripts share the same polyadenylation signal in exon 9. Thus, MDSP and TMDP are distinct proteins produced from the DUSP13 locus using alternative promoters.

MDSP and TMDP not only are both VH1-like DSPs but also belong to the same subgroup, of which the best characterized member is VHR. VHR, like many other VH1-like DSPs, is involved in MAP kinase signaling. Ten MAP kinase phosphatases (MKPs) contain an additional N-terminal domain specific for substrate (MAPK) recognition (22). In addition, other VH1-like DSPs including SKRP1 (47) and JSP1 (41, 48) were shown to be involved in regulation of Jnk signaling. MKP6 (49) and VHR have both been shown to target MAP kinases as substrates, although they lack the substrate recognition domain in the other MKPs. In particular, VHR has been shown to dephosphorylate preferentially the phosphotyrosine residue in ERK2 (3) although the kcat/Km of VHR toward ERK2 is 3 orders of magnitude lower than MKP3 in vitro (28). Unlike its close relative VHR, we were unable to detect any dephosphorylation of phospho-MAP kinases by MDSP in an overexpression system. Therefore, we concluded that MDSP is unlikely to function as a MAP kinase phosphatase.

The skeletal muscle-specific expression of MDSP suggested that its physiological substrate may also be a muscle-specific protein. The dual specificity tyrosine-regulated kinases (DYRKs) and MAPKs share sequence homology in their activation loops (50) and are involved in cell growth and development. DYRKs are activated by autophosphorylation of the second Tyr in the YXY motifs of their activation loops. Most interestingly, DYRK1B is involved in skeletal muscle development (51), and its p75 splice variant is only expressed in skeletal muscle (52); therefore, we tested whether MDSP expression decreased the level of phosphotyrosine in DYRKs. However, MDSP did not dephosphorylate DYRKs in a cotransfection system (data not shown), and thus DYRKs are also unlikely to be physiological substrates for MDSP.

Among other mammalian DSPs, homologues with apparently similar substrate specificities often showed different tissue distribution, but the highly restricted tissue expression patterns seen for MDSP and TMDP are uncommon. VHR, the closest characterized relative of both MDSP and TMDP, is constitutively expressed at high levels in a variety of mammalian cells (3, 30). In contrast, endogenous MDSP was only detected in skeletal muscle and, at lower levels, in muscle cells in culture, whereas the endogenous expression of TMDP is restricted to testis from mice <2 weeks old. The dramatic increase in MDSP expression during postnatal development of skeletal muscle suggests that MDSP may be involved in the late stages of muscle development. The molecular events orchestrating late stages of skeletal muscle development are less well characterized than those in myotube formation. However, it is known that many morphological and biochemical changes are required for the transition from myotube to mature myofiber, including fiber type switching, neuromuscular junction formation, and muscle size control. Most interestingly, the MDSP expression level in young mice (<2 weeks old) was comparable with that of the myotubes differentiated in culture. Skeletal muscles in 2-week-old mice are functional and have undergone many of the changes mentioned above, suggesting that the adult level of MDSP is not required in the myotube to myofiber maturation process, including neuromuscular junction formation and fiber type switching (53). Furthermore, we did not observe any difference in MDSP level among different muscle groups (data not shown), indicating that MDSP is not a fiber type marker (54). However, the increase in MDSP level correlated well with the increase in skeletal muscle mass in the developing quadriceps, suggesting a possible role of MDSP in muscle size control. Unfortunately, this expression pattern limits the availability of cell models and will force future analysis to encompass generations of animal models.

The gene structure of the DUSP13 locus was conserved between human and mouse, and probably also in F. rubripes. In contrast, the INK4a locus in Fugu does not encode the ARF protein (55). The close proximity of these DSP ORFs may be the result of a gene duplication event. Most interestingly, exons 4 and 5, which were not part of the coding regions of either MDSP or TMDP, were also well conserved between human and mouse. These conserved features across species are consistent with the functional significance of the unique gene structure. Both MDSP and TMDP were highly tissue-specific proteins; the former only detected in skeletal muscle and the latter only in testis. Moreover, the expression of both MDSP and TMDP proteins were up-regulated at approximately the same age during postnatal development in each tissue in mouse. Although the two DSPs were expressed from different promoters, the unusual arrangement of the DUSP13 locus may play a role in mediating the temporal coordination of MDSP and TMDP expression in development.

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Addendum—It has come to our attention that the cDNA sequence of human MDSP (GenBank™ accession number AY674051) has also been submitted to GenBank™ by Skurat and Dietrich under the name of BEDP, Branching Enzyme-interacting Dual specificity protein Phosphatase (GenBank™ accession number AY040091).

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Characterization of Two Distinct Dual Specificity Phosphatases Encoded in Alternative Open Reading Frames of a Single Gene Located on Human Chromosome 10q22.2

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