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A Novel Low-Molecular-Mass Dual-Specificity Phosphatase, LDP-2, with a Naturally Occurring Substitution That Affects Substrate Specificity[1]

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We have identified a novel dual-specificity phosphatase (DSP), called LDP-2 (low-molecular-mass DSP-2), composed of 220 amino acid residues showing high sequence homology to VHR and LDP-1/TMDP, which belong to a family of DSPs with low molecular masses. The LDP-2 gene is ubiquitously expressed, and LDP-2 is localized in the cytoplasm. The main structural feature of LDP-2 is that the serine-156 residue located in the common active site sequence motif, HCXXGXXRS, for DSP is naturally substituted with an alanine residue. The recombinant LDP-2 protein showed extremely low phosphatase activity towards *p*-nitrophenyl phosphate (pNPP). Back-mutation of Ala-156 in LDP-2 to a serine (A156S mutation) conferred significant phosphatase activity towards pNPP. However, both LDP-2 and LDP-2 (A156S) exhibited substantial phosphatase activities towards both phospho-seryl/threonyl and -tyrosyl residues of myelin basic protein, with similar specific activities. Ala-156 of LDP-2 might be crucially involved in the recognition of a physiological substrate. We analyzed the effect of VHR and LDP-2 on mitogen-activated protein kinases (MAPKs) *in vivo*. We first found that VHR inhibits the activation of p38 as well as ERK and JNK, with similar efficiency. Under the conditions ussed, LDP-2 specifically suppressed JNK activation.

Key words: dual-specificity phosphatase, low molecular mass, MAPK, natural substitution, substrate specificity.

Protein tyrosine phosphatases (PTPs), together with protein tyrosine kinases, control cell growth, differentiation, and activation through regulation of the levels of tyrosine-phosphorylation of proteins (*1*). PTPs are classified into two subclasses, tyrosine-specific PTPs and dual-specificity phosphatases (DSPs), which dephosphorylate both phosphotyrosyl and phosphothreonyl residues on their substrates. The DSP family includes: (i) MAPK phosphatases (MKPs), (ii) the CDC25 family, (iii) the CDC14 family, and (iv) other DSPs whose function is currently unknown (*2*). MKPs inactivate the MAPK family through dephosphorylation of the Thr-x-Tyr motif phosphorylated by the upstream MAPK kinase family *in vivo*. Several MKPs are inducible at the transcriptional level as a consequence of activation of the MAPK signaling pathway, suggesting that these enzymes may be involved in the negative feedback control (*3–5*). The CDC25 family and CDC14 family both contribute to the regulation of cell-cycle checkpoints (*6*). Recently, we reported a novel DSP, named TMDP, which is a DSP with a low molecular mass and abundant in the testis (*7*). VHR (*8*) is another DSP with a low molecular mass, and exhibits high sequence homology to TMDP. The catalytic mechanism of VHR has been well-characterized, but its physiological function remains unclear. Recently, the function of VHR in the MAPK cascades was reported. ERK1 and ERK2 were identified as proteins specifically absorbed to a VHR-affinity column, and both proteins were shown to be dephosphorylated by VHR *in vitro* and *in vivo* (*9*). In intact T lymphocytes, JNK as well as ERK were found to be substrates

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Abbreviations: DSP, dual-specificity phosphatase; LDP, low-molecular-mass DSP; pNPP, *p*-nitrophenyl phosphate; MAPK, mitogen-activated protein kinase; PTP, protein tyrosine phosphatase; MKP, MAPK phosphatase; TMDP, testis- and skeletal muscle–specific DSP; VHR, Vaccinia H1–related phosphatase; STYX, phosphoserine/threonine/tyrosine-binding protein; Sbf1, Suvar3-9 Enhancer-of-zeste Trithorax binding factor1; dbEST, expressed-sequence-tag database; RT, reverse transcriptase; GST, glutathione S-transferase; MBP, myelin basic protein; ERK, extracellular signal-regulated kinase; PRL-1, phosphatase of regenerating liver.

of VHR, but activation of p38 was not affected by VHR *in vivo* (*10*). More recently, an additional DSP with a low molecular mass, named LMW-DSP2, was identified. LMW-DSP2 was found to dephosphorylate JNK and p38 but not ERK (*11*). These data suggested that not only MKPs but also DSPs with low molecular masses might be involved in negative regulation of MAPK signaling.

The presence of several phosphatase-like proteins has been reported (*12*). These proteins do not possess phosphatase activity because of naturally occurring substitutions of catalytically essential residues. STYX (*13*) and MK-STYX (*12*) are related in amino acid sequence to VHR and MKPs, respectively, but lack intrinsic phosphatase activity due to the substitution of catalytic cysteine to glycine or serine. Sbf1 exhibits extensive sequence similarity with myotubularin, which is a DSP, and inhibits myoblast differentiation and also promotes anchorage-independent cell growth (*14*). The invariant cysteine and arginine residues in the catalytic domain active site of DSP are naturally substituted with leucine and isoleucine, respectively, in that of Sbf1 (*14*). These proteins are proposed to be protective factors that protect the substrate from dephosphorylation during intracellular signaling mediated by protein phosphorylation, rather than simply being non-functional homologs of active phosphatases (*12*).

In this study, we have isolated and characterized a novel and unique DSP with a low molecular mass, named LDP-2 (low-molecular-mass DSP-2). The structural feature of LDP-2 is that the serine-156 residue in the active site motif conserved among DSPs is naturally substituted with alanine. The recombinant LDP-2 exhibits very low activity towards pNPP compared with other DSPs. The A156S mutant of LDP-2, LDP-2 (A156S), gained the activity to a level comparable with in the cases of other DSPs. However, the activity towards a myelin basic protein (MBP) phosphorylated at a tyrosine or serine/threonine residue was almost the same for between LDP-2 and LDP-2 (A156S). In order to find physiological substrates for LDP-2, we compared the substrate specificities of VHR and LDP-2 *in vivo* using MAPKs (ERK, JNK, and p38) as substrates. We first demonstrated that VHR inhibits the activation of p38 as well as ERK and JNK, with similar efficiency, *in vivo*. Under the conditions used, LDP-2 was highly specific for JNK.

MATERIALS AND METHODS

cDNA Cloning of LDP-2—The expressed-sequence-tag database (dbEST) was screened using a nucleotide sequence of mouse LDP-1/TMDP (*7*) as a probe. Several clones were found to be highly similar to the C-terminal region of mouse LDP-1/TMDP on the screening. These cDNA clones (accession nos. AA216840 and AA921440) were obtained from Research Genetics (Huntsville, AL), and their nucleotide sequences were determined. The nucleotide sequences of dbEST clones were confirmed by analyzing mouse cDNA fragments isolated from mouse testis by reverse transcriptase (RT)-PCR using primers, 5′-TGAGCTTGCTGCTTTTCCTTC-3′ and 5′-GGTAACGTTCATTTCCACTGG-3′, according to the sequence of the dbEST clones described above. The first strand cDNA was synthesized with SuperscriptII (GIBCO BRL) using oligo(dT) as a primer. Total RNA from mouse testis was used as a template. The amplified 819-bp fragment was cloned into the pCRII vector (Invitrogen) and then sequenced. This clone identified on the screening was named LDP-2. The human LDP-2 cDNA was also obtained by RT-PCR using primers, 5′-CCGTTTTCTATGCCTGCTGG-3′ and 5′-TCAAATTGACCTCCATTTATGC-3′, according to the sequences of the dbEST clones (AI031656 and AI394036) from HL-60 cells.

Mutagenesis In Vitro—Ala-156 in an active site of LDP-2 was mutated to serine (LDP-2 (A156S)) by PCR-based mutagenesis, as previously described (*15*). For mutagenesis of Asp-118, primary PCR was performed using the cDNA corresponding to the entire coding region of LDP-2 as a template and two sets of primers: set 1, 5′-GCGGATCCATGCACTCCCTGAACCAAGAAATCAAAGC-3′ (primer 1) and 5′-ACAATTGCAGCAGACCTGGAAACAC-3′, and set 2, 5′-GTGTTTCCAGGTCTGCTGCAATTGT-3′ and 5′-GCGAATTCGATCACAGACCACCGGTCG-3′ (primer 2). Secondary PCR was performed using primer 1 and primer 2, and two kinds of primary PCR products as templates to obtain a full-length cDNA of the mutated LDP-2. The underlining indicates the substituted nucleotides. The final PCR products were cloned into pCRII and sequenced. No substitution was found except for the targeted mutation.

Bacterial Expression of the Recombinant Protein—For bacterial expression of LDP-2 and the mutants as glutathione S-transferase (GST)-fusion proteins, the coding regions of wild-type LDP-2 and LDP-2 (A156S) in pCRII described above were digested with *Bam*HI and *Eco*RI, and then subcloned into the corresponding sites of pGEX-6P-3 (Pharmacia). Expression and purification of GST-fusion proteins were performed as described previously (*7*).

Preparation of Substrates and Phosphatase Assay—MBP (Sigma), phosphorylated at either seryl/threonyl or tyrosyl residues, and pNPP were used as substrates, as described previously (*16*).

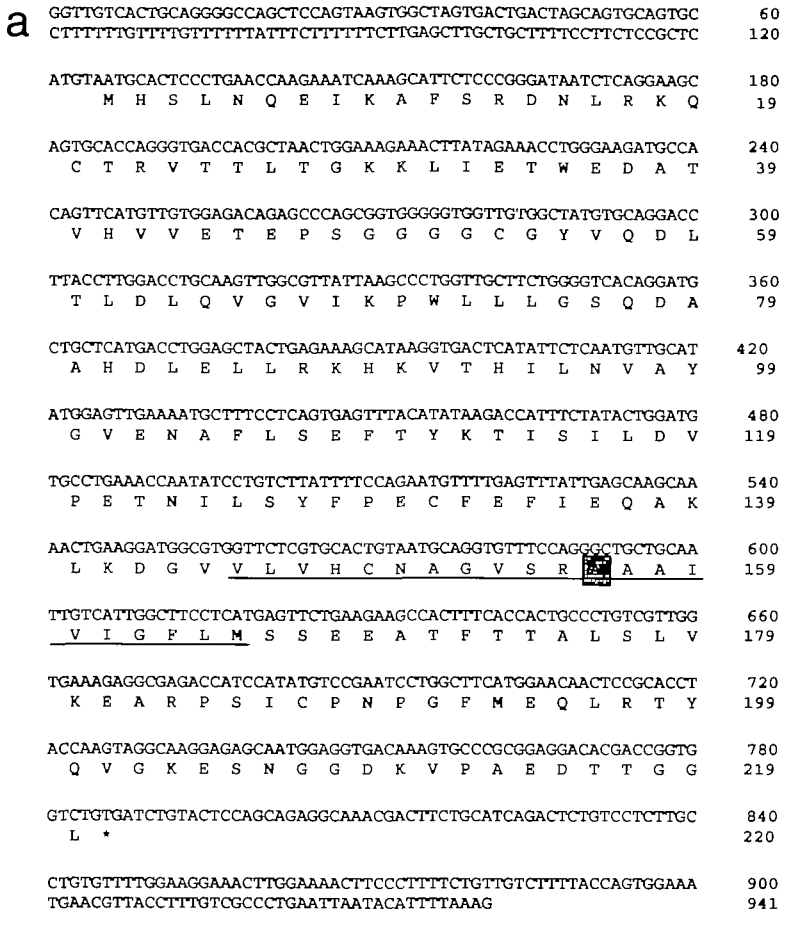
Northern Blot Analysis—Samples of 5 μg poly $(A)^+$ RNA from mouse tissues were separated on a 1% agarose gel containing 17% formaldehyde, blotted onto nitrocellulose membranes (Schleicher & Schuell), and then fixed by UV irradiation. The probe used for hybridization was a ^{32}P-labeled 663-bp fragment (nucleotides 33-696 in Fig. 1a) of LDP-2 cDNA.

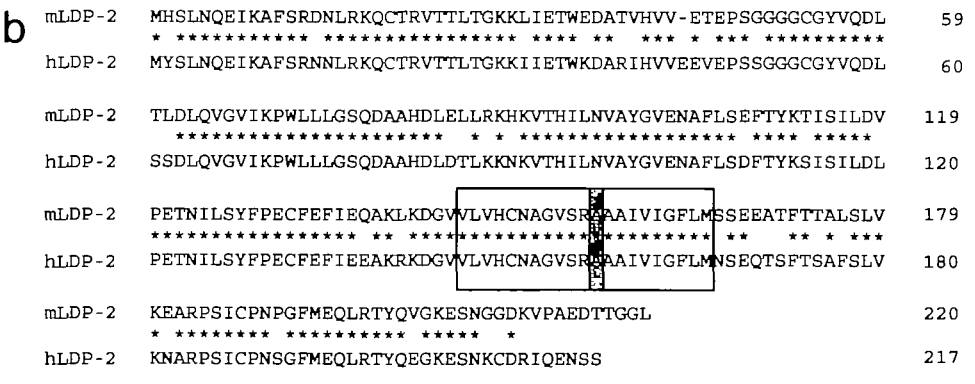
Plasmid Construction for Mammalian Expression—To construct pFLAG-LDP-2, pFLAG-LDP-2 (A156S), and pFLAG-VHR, the corresponding human cDNAs were amplified by PCR and subcloned to the pFLAG-CMV2 vector (Sigma, Buchs, Switzerland) in frame with the FLAG-epitope sequence. The PCR product was sequenced and then it was confirmed that no misincorporation was introduced.

Cell Culture and Transient Transfection—COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. Cells were co-transfected with pFLAG-LDP-2 or pFLAG-VHR together with SRα-HA-ERK2, SRα-HA-JNK1, or pMT3-HA-p38α. For transient assays, cells were transfected using Fugene-6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendation. Eighteen hours after transfection, cells were maintained with or without serum for 18 h, and then stimulated with either 25 ng/ml EGF for 20 min for ERK2 activation or with 0.4 M sorbitol for 30 min for JNK1 and p38α activation.

Detection of Activated MAPKs by Immunoblotting—

Transfected COS-7 cells were lysed in MAPK-lysis buffer [20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.5% deoxycholate, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 50 mM beta-glycerophosphate, 1 mM sodium vanadate, 10 mM





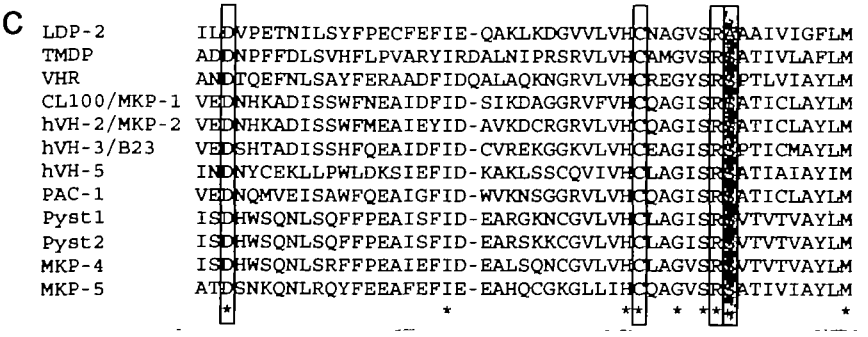


Fig. 1. **Nucleotide sequence of LDP-2 cDNA and the deduced amino acid sequences.** (a) The nucleotide sequence covering the entire coding region and the deduced amino acid sequence of mouse LDP-2 are shown. The termination codon is idicated by an asterisk. The active site motif conserved in dual-specificity phosphatases is underlined. The naturally occurring alanine substitution is shown by the shaded box. (b) Amino acid alignment of mouse and human LDP-2 is shown. Identical residues are indicated by asterisks. The catalytic domains conserved among DSPs are shown by the open box. The alanine substitutions are shown by the shaded box. The single letter amino acid code is used. Alignment of the amino acid sequences of the catalytic domains of DSPs. (c) The alignment of the amino acid sequences of the catalytic domains of mouse LDP-1, mouse TMDP (*7*), and other human DSPs, VHR (*8*), CL100/MKP-1 (*22*), hVH-2/MKP-2 (*23, 24*), hVH-3/B23 (*25, 26*), hVH-5 (*27*), PAC-1 (*28*), Pyst1 (*29*), Pyst2 (*29*), MKP-4 (*30*), and MKP-5 (*31*), is shown. The catalytic amino acid residues, aspartic acid, cysteine, and arginine, are indicated by open boxes. The amino acid residues conserved in all DSPs are indicated by asterisks. The gaps are shown by dashes. The alanine substituted in LDP-1 and the serine conserved in other DSPs are shown by the shaded box. The single letter amino acid code is used.

NaF, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin]. The cell lysates were centrifuged at 14,000 ×*g* for 15 min, and the resulting supernatants were used as cell extracts. Each sample (30 μg of protein) was separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and then transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). The phosphorylation status of activated MAPKs was monitored with an anti–phospho-ERK antibody (New England Biolabs/NEB, Beverly, MA, USA), an anti-ACTIVE JNK antibody (Promega, Madison, WI, USA), or an anti–phospho p38 antibody (NEB), followed by an HRP-conjugated donkey anti–rabbit IgG secondary antibody (CHEMICON International, Tumecula, CA, USA). The expression level of HA-tagged MAPKs, FLAG-tagged LDP-2, and FLAG-tagged VHR was monitored with an anti-HA (12CA5) monoclonal antibody (Roche Diagnostics) and anti–FLAG M2 monoclonal antibody (Sigma), respectively, followed by an HRP-conjugated rabbit anti–mouse IgG secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Signals were detected by enhanced chemiluminescence using the ECL reagent (Amersham Pharmacia Biotech).

Cell Staining—HeLa cells were transfected with pFLAG-LDP-2, and then fixed in PBS containing 3.7% formaldehyde for 10 min, permeabilized with PBS containing 0.25% Triton X-100 for 10 min, and then washed with PBS three times. After blocking with 3% BSA in PBS for 2 h at room temperature, the cells were incubated with anti-FLAG M2 antibodies (Sigma) in 3% BSA in PBS overnight at 4°C, washed with PBS three times, and then incubated with FITC-conjugated anti–mouse IgG secondary antibodies (Chemicon) in 3% BSA in PBS for 30 min at 37°C. After three washes with PBS, the coverslips were mounted with 90% glycerol in PBS. Fluorescence was visualized under a fluorescence confocal microscope (Olympus).

RESULTS

Identification of a Novel Dual-Specificity Phosphatase LDP-2—To search for a new member of the dual-specificity phosphatase (DSP) family exhibiting similarity with mouse TMDP, which is predominantly expressed in the testis (7), we screened the expressed sequence tag database (dbEST) with the nucleotide sequence of mouse TMDP as a probe. The cDNA sequences of the mouse dbEST clones (accession nos. AA216840 and AA921440) were found to be novel and to contain a partial open reading frame containing the active site for DSPs. These clones were obtained from Research Genetics (Huntsville, AL), and the entire nucleotide sequences were determined and found to completely overlap. The sequences of the novel mouse DSP were confirmed by sequencing the RT-PCR fragments isolated from mouse brain. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 1a. The methionine codon (nucleotides 126–128) was concluded to be the translation-initiation site, since it was preceded by an in-frame termination codon. The entire open reading frame comprised 660-bp and encoded a protein of 220 amino acid residues with a predicted molecular mass of 24.2 kDa. The deduced amino acid sequence of the novel DSP contained the active site motif of the DSP, VXVHCXAGXSRSXTXXXAY(L/I)M (where X is any amino acid) (Fig. 1a, underlined), with a

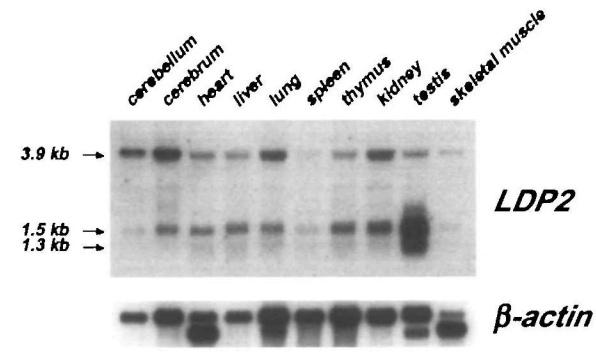


Fig. 2. **Expression of LDP-2 mRNA in various mouse tissues.** Poly (A)$^+$ RNA (5 μg) obtained from various mouse tissues, as indicated, was separated on a 1% agarose gel, transferred to a nitrocellulose membrane, and then hybridized with a random-primed ^{32}P-labeled probe for *LDP-2*. The sizes of LDP-2 transcripts are indicated on the left. The membrane was reprobed with *β-actin* as an internal control.

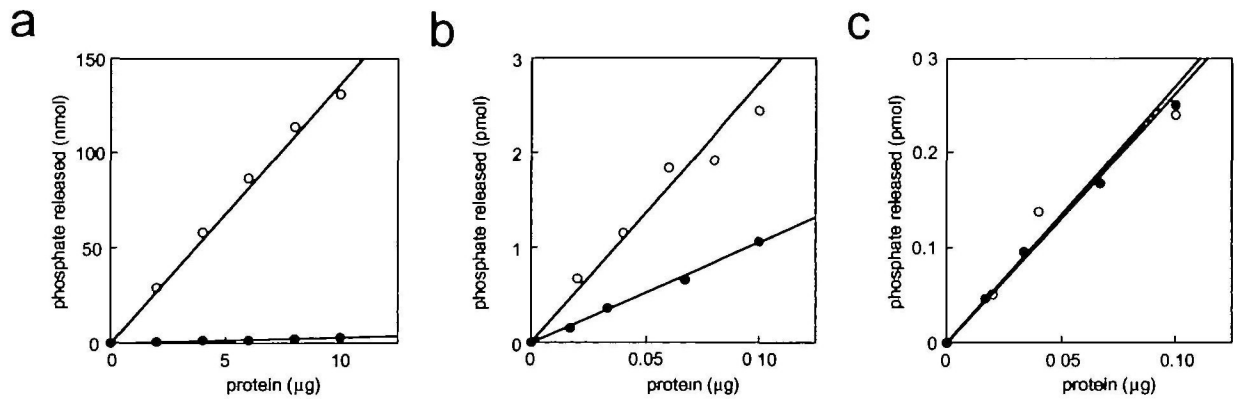


Fig. 3. **Phosphatase activities of recombinant LDP-2 and mutant LDP-2 (A156S).** The phosphatase activities of both LDP-2 (closed circles) and LDP-2 (A156S) (open circles) fused to GST were determined using pNPP and MBP as substrates with an incubation time of 10 min at 30°C. (a) Dephosphorylation of 10 mM pNPP by increasing amounts of LDP-2 and LDP-2 (A156S). (b) Dephosphorylation of 2 mM Tyr (P)-MBP by increasing amounts of LDP-2 and LDP-2 (A156S). (c) Dephosphorylation of 2 mM Ser/Thr (P)-MBP by increasing amounts of LDP-2 and LDP-2 (A156S). The data shown are the averages of at least two independent experiments.

substitution at 156 from serine to alanine. Importantly, the three amino acid residues essential for the catalytic activity of DSPs were conserved in LDP-2 at Cys-149, Arg-155, and Asp-118 (Fig. 1a). Comparison of the amino acid sequence of the novel DSP with the Genbank/EMBL Data Bank revealed that the similarity to other DSPs was restricted within the catalytic domain, being 45 and 40% to VHR and TMDP, respectively. During EST-based cDNA cloning, we identified 6 different DSPs with low molecular masses, including TMDP and this novel DSP. Then we renamed TMDP LDP-1 (low-molecular-mass dual-specificity phosphatase-1) and named the novel DSP LDP-2. A human LDP-2 orthologous gene was also identified using the dbEST library, and the amino acid sequence of human LDP-2 was shown with that of mouse LDP-2 in Fig. 1b. Human LDP-2 exhibits 83% identity with mouse LDP-2.

TABLE I. **Kinetic parameters of the LDP-2 and LDP-2 (A156S) enzymes.** Reactions were carried out at 30°C in 100 mM sodium acetate, pH 5.0, 1.6 mM dithiothreitol using pNPP as a substrate. The values shown are the averages of at least three separate experiments.

Enzyme	V_{max} (nmol/min/mg)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
LDP-2	33.5 ± 2.5	1.54 ± 0.60	0.029 ± 0.003	18.5 ± 9.2
LDP-2 (A156S)	1,040 ± 28	16.8 ± 4.8	0.881 ± 0.23	52.8 ± 9.1

The naturally occurring substitution from serine to alanine in mouse LDP-2 was also observed in human LDP-2 (Fig. 1, a and b). A catalytic domain of mouse LDP-2 was aligned with those of other DSPs, as shown in Fig. 1c.

Tissue Distribution of LDP-2 mRNA—The expression pattern of LDP-2 mRNA in mouse tissues was examined by Northern blot analysis of poly $(A)^+$ RNA using the entire coding region of mouse LDP-2 cDNA as a probe. As shown in Fig. 2, three mRNA transcripts of 3.9, 1.5 and 1.3 kb were detected in all tissues examined. The 3.9 kb transcript was expressed at high levels in the cerebrum, cerebellum, lung and kidney, whereas the 1.5 kb transcript was expressed in the testis at the highest level. The 1.3 kb transcript was observed dominantly in the testis.

Phosphatase Activities of Recombinant LDP-2 and LDP-2 (A156S)—To assess the effect of the naturally occurring substitution at 156 from serine to alanine on phosphatase activity and/or substrate specificity, the wild and mutated (A156S) types of LDP-2 were produced as GST-fusion proteins. GST-LDP-2 and GST-LDP-2 (A156S) were subjected to the phosphatase assay using pNPP as a substrate (Fig. 3a). Although GST-LDP-2 dephosphorylated pNPP in proportion to the enzyme amount added, the specific activity of GST-LDP-2 was 100 times lower than those of LDP-1/TMDP (*7*) and other DSPs (*17, 18*). The GST-LDP-2 (A156S) protein gained the catalytic activity towards pNPP and the specific activity of GST-LDP-2 (A156S) (1,340 nmol/

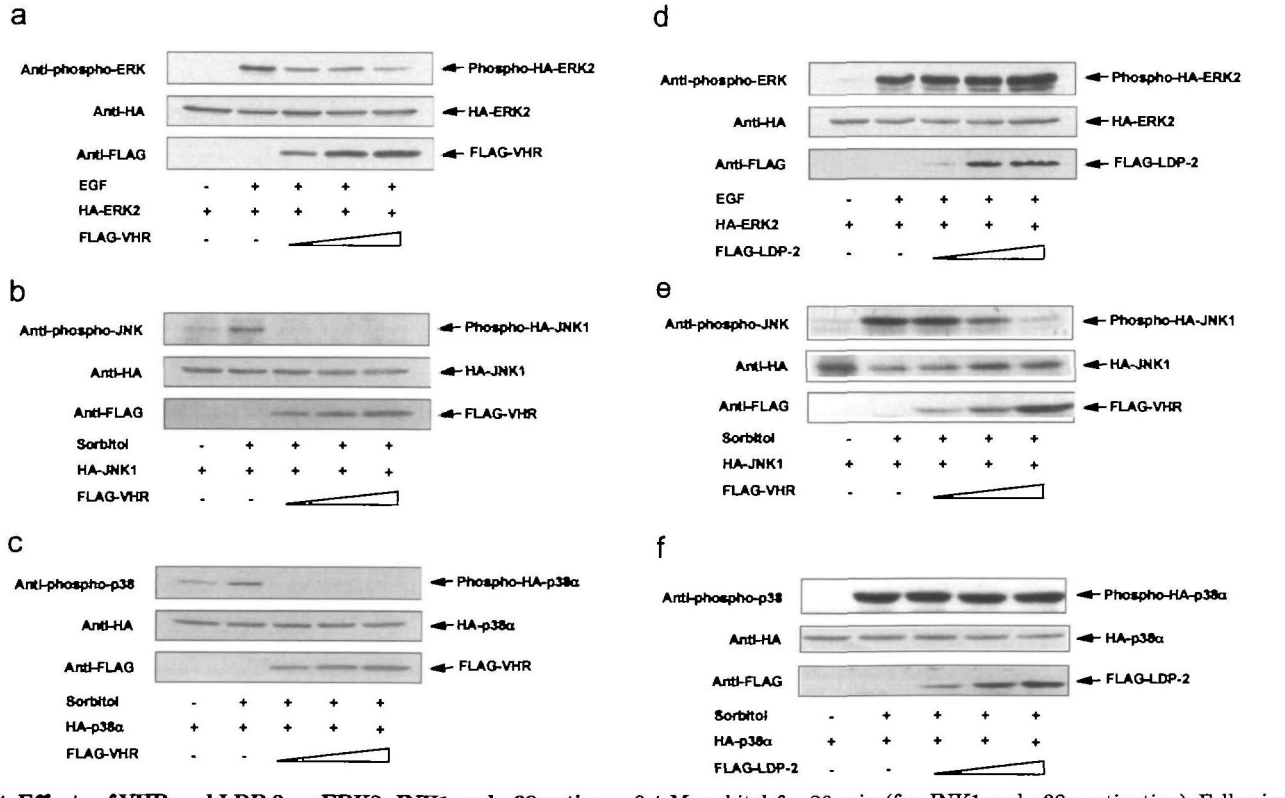


Fig. 4. **Effects of VHR and LDP-2 on ERK2, JNK1 and p38 activation.** COS-7 cells were co-transfected with 0.5 μg of an expression plasmid encoding HA-ERK2, HA-JNK1, or HA-p38 together with 0.375, 0.75, or 1.5 μg of pFLAG-VHR or pFLAG-pLDP-2. Transfected cells were incubated for 18 h in serum-free medium and then stimulated with either 25 ng/ml EGF for 20 min (for ERK2 activation) or 0.4 M sorbitol for 20 min (for JNK1 and p38α activation). Following cell lysis, the expression levels of HA-MAPKs, FLAG-DSPs (VHR and LDP-2) were monitored on immunoblots using anti-HA and anti-FLAG antibodies, respectively. Phosphorylated HA-ERK2, HA-JNK1, and HA-p38α were detected with anti–phospho-ERK, anti–ACTIVE JNK, and anti–phospho-p38 antibodies, respectively.

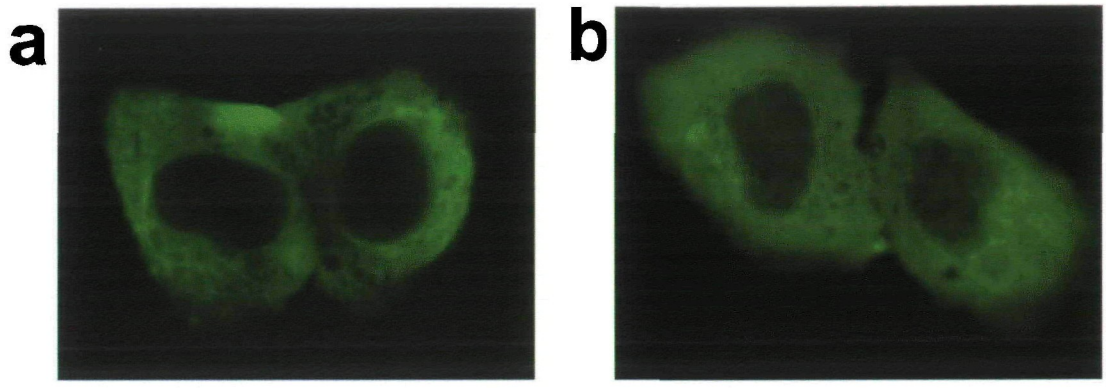


Fig. 5. **Subcellular localization of LDP-2.** HeLa cells were transfected with 2 μg of Flag-LDP-2 (a) or Flag-LDP-2 (A156S) (b). After 48 h, the cells were fixed and incubated with anti-FLAG antibodies (M2), followed by incubation with FITC-conjugated anti-mouse IgG and visualization under a fluorescence microscope.

min per mg) became 50 times higher than that of GST-LDP-2 (27.1 nmol/min per mg) (Fig. 3a). Thus, we concluded that the low activity of LDP-2 towards pNPP is due to the naturally occurring substitution of the serine residue to alanine. We also determined the phosphatase activities of GST-LDP-2 and GST-LDP-2 (A156S) towards tyrosine-phosphorylated MBP [Tyr(P)-MBP] or serine/threonine-phosphorylated MBP [Ser/Thr (P)-MBP]. Unexpectedly, both GST-LDP-2 and GST-LDP-2 (A156S) dephosphorylated Tyr (P)-MBP with linearity up to 0.1 mg under the conditions used (Fig. 3b). The specific activity of GST-LDP-2 towards Try (P)-MBP (1.06 nmol/min per mg) was lower than that of GST-LDP-2 (A156S) (3.00 nmol/min per mg) at the same order of magnitude and comparable with that of GST-LDP-1/TMDP (1.12 nmol/min per mg) (*7*). Furthermore, both GST-LDP-2 and GST-LDP-2 (A156S) also dephosphorylated Ser/Thr (P)-MBP with linearity up to 0.1 mg under the conditions used (Fig. 3c). The specific activities of GST-LDP-2 and GST-LDP-2 (A156S) were 0.247 and 0.241 nmol/min per mg, respectively. These results showed that Ala-156 contributes to the substrate specificity. The kinetic parameters with pNPP as a substrate are shown in Table I. The mutation of Ala-156 to serine increased k_{cat} and K_m 30-fold and 10-fold, respectively. The k_{cat}/K_m value was slightly increased (2.8-fold) by the mutation.

Dephosphorylation of MAPKs by VHR and LDP-2—VHR, to which LDP-2 exhibits the highest sequence homology, is the first identified DSP except for the MKP family to dephosphorylate MAPKs *in vivo*. We compared the effects of VHR and LDP-2 on the activation of HA-ERK2, HA-JNK1 or HA-p38α *in vivo*. As shown in Fig. 4, VHR effectively dephosphorylates all of HA-ERK2, HA-JNK1, and HA-p38α, demonstrating that VHR can dephosphorylate p38 in addition to ERK and JNK (*9, 10*) *in vivo*. Under the conditions used, LDP-2 did not affect the phosphorylation levels of HA-ERK and HA-p38, however, it effectively suppressed the activation of HA-JNK in a dose-dependent manner (Fig. 4). Such a substrate specificity of LDP-2 towards MAPKs was not influenced by the A156S substitution (data not shown). These data suggested that despite the high sequence homology to VHR, LDP-2 is highly specific for JNK, but A156 is not involved in JNK recognition.

Subcellular Localization of LDP-2—To determine the subcellular localization of LDP-2, HeLa cells were transiently transfected with Flag-tagged LDP-2. Indirect immunofluorescence with anti-Flag antibodies showed that Flag-LDP-2 is mainly distributed in the cytoplasm (Fig. 5a). The distribution of Flag-LDP-2 (A156S) or Flag-LDP-2 (D118A) was essentially the same as that of Flag-LDP-2 (Fig. 5b).

DISCUSSION

The present study demonstrated that LDP-2 (24.2 kDa) is a novel member of the low-molecular-mass DSP family that comprises VHR (20.5 kDa) (*8*), LDP-1/TMDP (22.5 kDa) (*7*), and LMW-DSP2 (20.2 kDa) (*11*). The structural feature of the primary structure of LDP-2 is that the serine residue in the active site conserved in DSPs is naturally substituted with alanine (Ala-156). CDC25 has a glycine residue instead of serine in the conserved active site sequence motif, but the other serine residue (position +3 from the catalytic cysteine) is thought to play the same role as the serine residue in the catalysis (*19*). PRL-1, a nuclear PTP with a molecular mass of 20 kDa, also lacks the invariant serine residue. Although the activity of PRL-1 towards tyrosyl-phosphorylated protein substrates has not been reported, its activity towards pNPP was much lower than those of other PTPs (*20*). These reports suggested that the serine is essential for the enzyme activity. Previously, Denu and Dixon introduced a mutation at the conserved Ser-131 in VHR and showed that the mutation of the invariant serine to alanine alters the rate-limiting step in the catalysis, and claimed that the serine residue conserved in all DSPs acts to stabilize the thiolate ion of the catalytically important cysteine, assisting in the second step of catalysis (*21*). The rate-limiting step for the catalysis of native VHR is the formation of an intermediate phosphoenzyme, whereas breakdown of the intermediate is rate-limiting for the S131A mutant in the assay with pNPP as a substrate (*21*). In present study with pNPP as a substrate, the specific activities of a native LDP-2 and a LDP-2 (A156S) mutant resembled those of the S131A mutant of VHR and the native VHR, respectively (*21*). However, the specific activities of LDP-2 and LDP-2 (A156S) towards Tyr (P)-MBP or Ser/Thr (P)-MBP were almost the same as those of LDP-1/TMDP (*7*) under the same assay conditions. Thus, these results demonstrated that the naturally occurring substitution in LDP-2 is not a phosphatase-dead mutation, and suggest

that Ala-156 in LDP-2 contributes to the substrate specificity *in vivo*.

In addition to VHR, LMW-DSP2, LDP-1/TMDP and LDP-2, we recently isolated several other members of the LDP family (data not shown). The LDP family is still expanding, but the physiological function of each member is not well understood. Recently, some members were shown to be involved in MAPK signaling through dephosphorylation of MAPK(s) although they lack the MAPK-docking motif conserved in MKPs (*9–11*). VHR was the first LDP to be determined to dephosphorylate ERK and JNK, but its effect on p38 was not clear. Here we clearly showed that VHR dephosphorylates p38 as well as ERK and JNK, with similar preference, using sorbitol as a stimulant for COS 7 cells. Under the same conditions, we showed that LDP-2 suppressed the JNK pathway specifically. At this point we can conclude that there are three types in the LDP family, first VHR, as a general MAPK phosphatase, second LMW-DSP2, as an ERK/JNK phosphatase (*11*), and third, LDP as a JNK phosphatase. Recently, we isolated MKP-7, which suppressed MAPK activation in COS-7 cells in the following order of selectivity, JNK ≫ p38 > ERK (*32*). Further experiments focusing on both LDP-2 and MKP-7 will answer the question how the JNK signal is regulated in the cells.

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