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Differential expression of two distinct forms of mRNA encoding members of a dipeptidyl aminopeptidase family

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ABSTRACT **We have identified two cDNAs encoding dipeptidyl aminopeptidase-like proteins (DPPXs) in both bovine and rat brains that have different N-terminal cytoplasmic domains but share an identical transmembrane domain and a long C-terminal extracellular domain. In both species, one of the cDNAs encodes a protein (designated DPPX-S) of 803 amino acid residues with a short cytoplasmic domain of 32 amino acids, and the other cDNA encodes a protein (designated DPPX-L) with a longer cytoplasmic domain—the bovine cDNA encodes 92 amino acids and the rat cDNA encodes 88 amino acids. The membrane topology of DPPX-S and -L is similar to that of other transmembrane peptidases, and DPPXs share ≈30% identity and 50% similarity with reported yeast and rat liver dipeptidyl aminopeptidase amino acid sequences, suggesting that DPPX is a member of the dipeptidyl aminopeptidase family. DPPX-S mRNA is expressed in brain and some peripheral tissues including kidney, ovary, and testis; in contrast, DPPX-L mRNA is expressed almost exclusively in brain. No transcripts for either form are found in heart, liver, or spleen. *In situ* hybridization studies show that the two transcripts have different distributions in the brain. DPPX-L mRNA is expressed in limited regions of brain with the highest level of expression in the medial habenula. More widespread expression is seen for DPPX-S mRNA. The differential distribution of mRNAs for the DPPX-S and -L suggests that these proteins are involved in the metabolism of certain localized peptides and that the cytoplasmic domain may play a key role in determining the physiological specificity of DPPX.**

Various peptides in the central nervous system function as neurotransmitters, neuromodulators, or hormones (1). Certain peptidases regulate the biological activities of these peptides by converting precursors to active forms or active forms to inactive forms (2–4). Dipeptidyl aminopeptidases (DPPs; EC 3.4.14) make up one group of these peptidases that appear to play a crucial role in the activation and inactivation of such peptides (3). Previous biochemical studies found that at least four distinct enzyme proteins (DPPI–IV) account for DPP activity in mammalian tissues based on substrate specificity (3). However, recent molecular cloning of a cDNA for a rat liver DPPIV (EC 3.4.14.5) and immunological characterization of DPPIV have suggested that several proteins are members of the DPPIV class (5–7). Some reports have suggested additional forms of DPP besides DPPI–IV (8–10). The presence of both soluble and membrane-associated forms of DPPs is another reason for the confusion about the classification of DPPs (3, 6, 9). Molecular biological approaches will extend the characterization and classification of DPPs and their activities.

We have isolated a cDNA encoding a DPP-like protein during our characterization of minor components in a purified protein fraction from bovine brain. We solubilized and purified an α-amino-3-hydroxy-5-methylisoxazole-4-propionate subtype of a glutamate receptor in bovine brain (11). Several peptide sequences were obtained from the protease-treated fragments of the purified protein. A bovine cDNA library was screened with oligonucleotide probes designed from the peptide sequences. Sequence analysis of positive phage clones revealed that some of the peptide sequences were encoded by a cDNA unrelated to the reported α-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor cDNAs (12, 13). The predicted protein (designated DPPX)§ encoded by the cDNA shows highest similarities to amino acid sequences of a yeast DPPB (14) and a rat liver DPPIV (5, 6), suggesting that the protein is a member of the DPP family. Further analysis using rat homologues and the bovine cDNA indicates that there are two distinct cDNAs (designated DPPX-S for the short form and DPPX-L for the long form) that are probably produced by an alternative splicing of RNA. Southern blot hybridization of PCR products and *in situ* hybridization show that the expression of the two alternative transcripts is regulated both in tissue- and region-specific manners.

MATERIALS AND METHODS

Peptide Sequences. An α-amino-3-hydroxy-5-methylisoxazole-4-propionate subtype of a glutamate receptor was solubilized and purified from bovine brain through a series of chromatographic steps (11). Proteolytic fragments of the protein were prepared as described (15) and subject to microsequencing. Several peptide sequences were obtained and one of them was used to make oligonucleotide probes, MAKVASRVSALEGQXFLVIXA, where X is an unidentified residue.

Library Screening. By using a codon frequency table (16), the following two long oligonucleotides of 57 bases encoding the peptide sequence above were made: 5′-ATGGCCAAGGTGGCCTCCMGMGTGTCCGCCCTGGAGGGCCAGTGCTTCCTGGTGATC-3′ and 5′-ATGGCCAAGGTGGCCAGCMGMGTGAGCGCCCTGGAGGGCCAGTGCTTCCTGGTGATC-3′, where M is A or C. The oligonucleotides were 5′-end-labeled with ^{32}P (17) and used to screen a bovine brain cDNA library (a gift from R. Huff, Upjohn; ref. 18) constructed in the λZAP vector (Stratagene). Hybridization and washing of filters were carried out according to a standard method (17). One of the positive clones was further used to screen a rat brain cDNA library (a gift by J. Boulter,

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Abbreviations: DPP, dipeptidyl aminopeptidase; DPPX, DPP-related protein; DPPX-S, short form of DPPX; DPPX-L, long form of DPPX.

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§The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M76426 (rat DPPX-L), M76427 (rat DPPX-S), M76428 (bovine DPPX-L), and M76429 (bovine DPPX-S)].

Salk Institute) constructed in the λZAP vector to isolate rat homologues. Isolated phage clones were excised *in vitro* to form phagemids according to a manufacturer's manual. Double-strand DNA of phagemids was sequenced on both strands by a dideoxynucleotide method using various synthetic primers (19).

Southern Blot Analysis of PCR Products. Poly(A)$^+$ RNAs from rat brain, heart, kidney, liver, ovary, pancreas, prostate, spleen, and testis were purchased from Clontech. First-strand cDNAs were synthesized from 1 μg of the poly(A)$^+$ RNAs from each tissue using oligo-(dT) primers. One common antisense and two specific sense oligonucleotides were made using rat nucleotide sequences to amplify a specific form of DPPX cDNA (positions 1208–1230 and positions 91–109 in the nucleotide sequence for rat DPPX-S and positions 416–434 in the sequence for rat DPPX-L, respectively). The PCR (20) was performed using 10% of the synthesized first-strand cDNA as a template and each set of sense and antisense oligonucleotides as primers. Each PCR cycle included a 2-min annealing reaction at 54°C, a 2.5-min extension at 72°C, and a 1-min denaturation at 94°C. After 30 cycles of amplification, 10% of the PCR products were fractionated on a 1% agarose gel and transferred onto a nylon membrane. As positive controls, PCRs using the isolated rat cDNAs as templates with the same primers were performed. Fragments amplified by the control PCRs were labeled with ^{32}P and used as probes. Filters were hybridized overnight in 5× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA) at 65°C, washed in 0.2× SSPE at 65°C, and exposed to an x-ray film for 5 h. Patterns of signals were not changed after longer exposures. To evaluate the quality of synthesized cDNAs, the same amount of the cDNAs was amplified by the PCR with a set of 20-base primers for rat β-actin cDNA (positions 1246–1265; positions 3110–3129 in a deposited sequence, GenBank accession no. J00691). Southern blot hybridization of the control β-actin products was performed as described above using a ^{32}P-labeled human β actin as a probe (a gift of J. Battey, National Institutes of Health). In each PCR, amplification without template cDNA was performed as a negative control.

***In situ* Hybridization.** *In situ* hybridization histochemistry was performed on freshly frozen rat brain sections (16 μm) by a modification of the method as described (15). Two 48-base oligonucleotides complementary to the nucleotide sequences of rat DPPX-S and -L cDNAs (positions 220–267 in the deposited rat DPPX-S sequence and 198–245 in the deposited rat DPPX-L sequence) were ^{35}S-labeled and used as probes. After hybridization, slides were washed in 1× standard saline citrate at 56°C, dehydrated, and exposed to x-ray films for 4 days. For higher-resolution studies, slides were dipped in Kodak NTB-2 nuclear emulsion and exposed at 4°C for 3 weeks.

RESULTS

Isolation and Characterization of cDNA Clones. Four overlapping clones (BQ723, BQ729C, BQ730C, and BQ735C) were isolated from about 3 × 10^6 phages and sequenced (Fig. 1). A fragment (*Apa* I–*Bam*HI) of the cDNA insert of BQ729C was also used as a probe to isolate rat homologues from a rat brain cDNA library. Three rat clones (RQ766A, RQ766D, and RQ772D) were isolated and further studied. Sequence analyses of these clones revealed two open reading frames that shared identical 3′ sequences but had different 5′ sequences in each species. The shorter open reading frames for DPPX-S are 2409 bases long in bovine and rat and show 84% identity between the two species at the nucleotide level. The longer reading frames for DPPX-L are composed of 2589 and 2577 bases in bovine and rat, respectively, and share 85% nucleotide identity between the two species. DPPX-S and -L

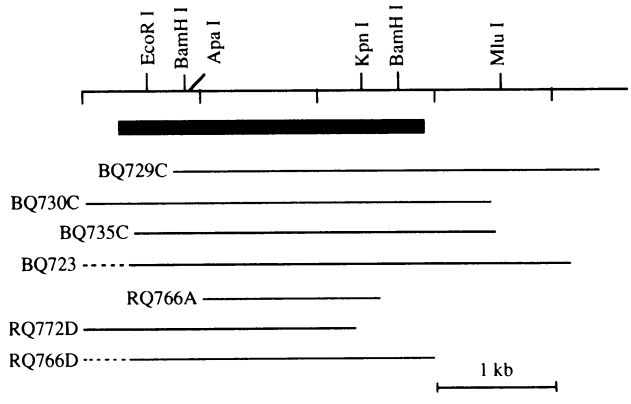

FIG. 1. Map of bovine and rat cDNAs encoding DPPX-S and -L. Solid box represents the protein-coding region of DPPX-S. Broken lines show the regions encoding the part of DPPX-L that is different from DPPX-S. Restriction enzymes for bovine DPPX-S cDNA are shown. kb, Kilobase.

cDNAs share 2403 bases in 3′ region in bovine and rat. The sequences around the predicted initiator methionine codon in both frames agree with the consensus sequence described by Kozak (21) in bovine and rat.

Analyses of Predicted Protein Sequences. Bovine and rat DPPX-S cDNAs encode 803 amino acid residues with a calculated molecular mass of 91 kDa without glycosylation. Bovine DPPX-L cDNA encodes 863 amino acids and rat DPPX-L cDNA encodes 859 amino acids (97 kDa without glycosylation in both species). Between the two species, DPPX-S and DPPX-L share 92% identity and 95% similarity, respectively. A homology search of sequence banks (NBRF, Swiss-Prot, GenBank, and EMBL) revealed that the DPPXs show the highest similarity to yeast (14) and rat liver (5, 6) DPPs. Both forms of DPPXs share 27% and 33% identity and 48% and 53% similarity (including conserved amino acid residues) with the yeast and rat liver DPPs, respectively. No striking homology was found with any other proteins including various other peptidases or proteinases. Dot plots of matrix analyses (22) of the amino acid sequence homology of DPPXs to a rat liver DPPIV and a yeast DPPB showed a diagonally linear line in most of the compared regions in each analysis, confirming that these three genes are evolutionarily related (data not shown). Fig. 2 shows the alignment of bovine and rat DPPX-S amino acid sequences with the reported rat liver DPPIV sequence (5, 6). Analysis of regional hydrophobicity (24) of DPPX-S and -L revealed one putative transmembrane domain near the N terminus. The membrane topology is similar to those of transmembrane peptidases (25), suggesting that the short N-terminal segment is in the cytoplasm and that the long C-terminal segment is extracellularly located. DPPX-S and DPPX-L have identical transmembrane domains and long C-terminal extracellular domains but have different sequences in most of the N-terminal cytoplasmic domain. In the C-terminal extracellular domain, bovine DPPX has six potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr where Xaa is any residue, except probably, proline and aspartic acid, see ref. 23), which are also conserved in the rat DPPX.

Tissue Distribution of DPPX-S and DPPX-L mRNAs. Northern blot analysis using the rat DPPX cDNA probe on mRNAs from various rat tissues showed that only brain expressed the transcripts of 3.8 and 4.5 kilobases (data not shown). However, use of a more sensitive method that combines the PCR and Southern blot analysis showed that DPPX-S mRNA was also expressed in peripheral tissues including kidney, ovary, prostate, and testis (Fig. 3). In contrast, DPPX-L mRNA was almost exclusively expressed in brain. Very faint signals for DPPX-L mRNA were also detected in ovary and prostate.

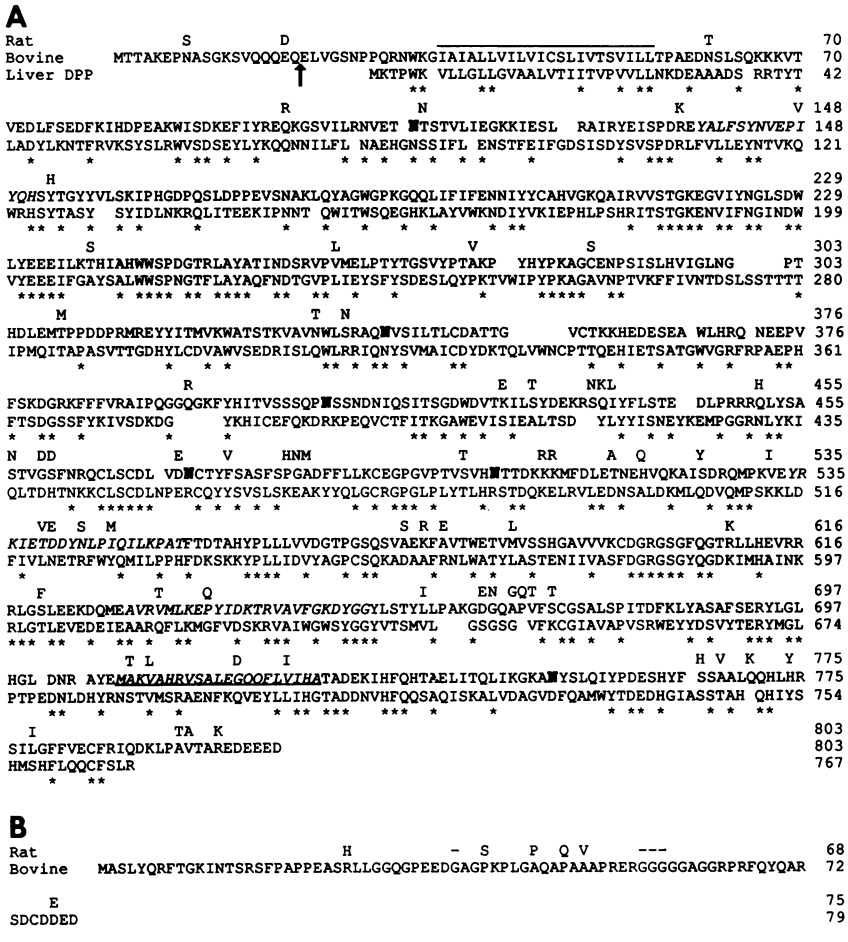

FIG. 2. (*A*) Amino acid sequences (single-letter code) of rat and bovine DPPX-S proteins and rat liver DPPIV (5, 6). (*B*) Bovine and rat DPPX-L amino acid sequences. Only the segment that differs from the DPPX-S amino acid sequence is shown. In *A* and *B*, only the residues in rat sequences that differ from the bovine sequence are shown. In *A*, identical residues in the three sequences are indicated by asterisks. Gaps are inserted to achieve maximum similarities. The arrow indicates where DPPX-S and -L start to share an identical amino acid sequence. A predicted transmembrane domain is indicated by the bar above the bovine sequence. Amino acid residues in italic type show chemically obtained peptide sequences. Among them, the peptide sequence used to make oligonucleotide probes was underlined. Outlined asparagine residues show potential N-linked glycosylation sites (23). In *B*, dashes show gaps in the sequence.

No transcripts for either DPPX-L or -S were found in heart, liver, or spleen. In pancreas, we could not confirm the absence of DPPX transcripts because of the low amplification of cDNA for control actin.

Differential Localization of DPPX-S and DPPX-L mRNAs in Rat Brain. *In situ* hybridization using specific probes for both DPPX-S and -L mRNAs revealed that the two transcripts differ markedly in their patterns of expression within many regions of the brain. In general, DPPX-L mRNA showed more limited localization than DPPX-S mRNA. In olfactory regions, moderate to high expression of DPPX-S transcripts was seen in the anterior olfactory nucleus and in the mitral cell layer of the main olfactory bulb. In contrast, signals were undetectable for the DPPX-L probe in these regions (Fig. 4 *A* and *B*). High levels of DPPX-S mRNA were present in the hippocampal complex, but DPPX-L mRNA was expressed at very low levels in these regions (Fig. 4 *C* and *D*). The medial habenula showed very intense signals with DPPX-L probe, but DPPX-S probe showed lower signals in the region.

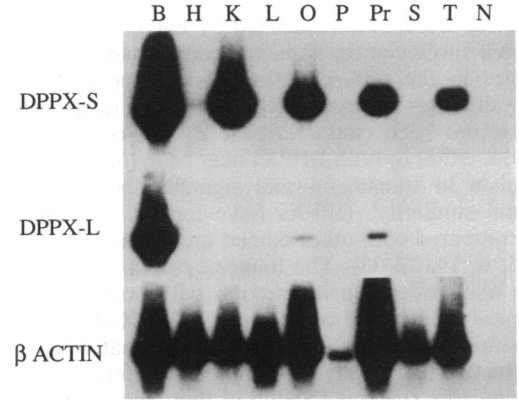

FIG. 3. Tissue distribution of DPPX mRNAs analyzed on a Southern blot of PCR products. Lanes: B, brain; H, heart; K, kidney; L, liver; O, ovary; P, pancreas; Pr, prostate; S, spleen; T, testis; N, negative control. Because of degradation of the RNA sample, poor amplification was observed in pancreas.

DISCUSSION

The predicted proteins (DPPX-S and -L) show the highest amino acid sequence similarity to yeast DPPB and a rat liver DPPIV. Sequence comparison of both DPPs and DPPX with other known proteins including peptidases and proteases did not reveal any significant homology. The amino acid sequences of the three proteins DPPB, DPPIV, and DPPX share about 30% identity and 50% similarity. This homology indicates that DPPX is a member of the DPP family. In mammals, DPP has been shown to have at least four subtypes (DPPI through -IV) delineated by their substrate specificity (3). Since DPPIV (and also yeast DPPB) shows a preference for peptides when the second residue is proline (6, 14), Gly-Pro-*p*-nitroanilide is often used as an artificial substrate for DPPIV (26). However, preliminary results have shown that both membrane and cytosolic fractions of simian kidney cells (COS-7) transfected with expressible cDNAs for DPPX-S and -L did not show enzymatic activity to this artificial substrate (N.Y., K.D., and K.W., unpublished observations). Other artificial substrates that are often used to characterize DPPI through -III (3, 10) were also not substrates for the expressed proteins in transfected cells. These results suggest that the DPPXs are functionally distinct from classical DPPs.

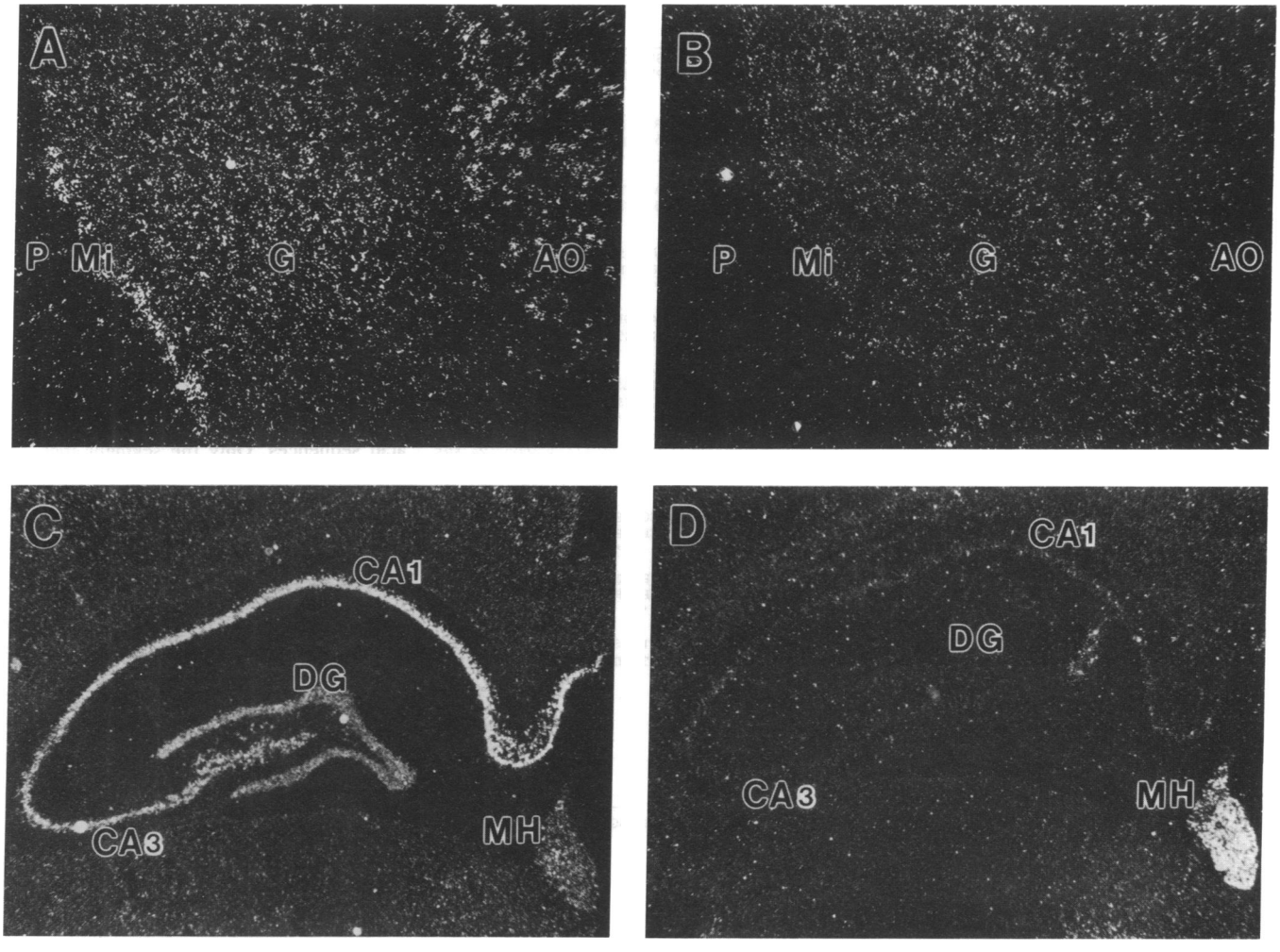

FIG. 4. Distribution of DPPX-S (*A* and *C*) and -L (*B* and *D*) mRNA. Darkfield photomicrographs in the olfactory bulb (*A* and *B*) and in the hippocampus regions (*C* and *D*) are shown. AO, anterior olfactory nucleus; G, granular layer; Mi, mitral cell layer; P, external plexiform layer; CA1, field CA1 of Ammon's horn; CA3, field CA3 of Ammon's horn; DG, dentate gyrus; MH, medial habenula.

Analysis of hydrophobicity of both DPPX-S and -L indicates that both forms have a membrane topology similar to those of the following cloned transmembrane hydrolases on cell surface; rat liver DPPIV (5, 6), yeast DPPB (14), γ-glutamyl transpeptidase (EC 2.3.2.2.) (27), sucrase-isomaltase (EC 3.2.1.48 or 10) (28), neutral endopeptidase (EC 3.4.24.11) (29), aminopeptidase N (EC 3.4.11.2) (30), aminopeptidase A (EC 3.4.11.7) (31, 32), and renal dipeptidase (EC 3.4.13.11) (33). Each enzyme consists of a short N-terminal cytoplasmic domain, a transmembrane domain composed of a stretch of hydrophobic amino acid residues, and a long C-terminal extracellular domain. Previous biochemical studies on these enzymes demonstrated that catalytic domains are located in the long C-terminal extracellular domain (25). Based on such a model, since both DPPX-S and -L share an identical extracellular segment, the mode of the potential enzyme activity of the two proteins appears quite similar if not identical. However, Southern blot analysis of PCR products and *in situ* hybridization show that DPPX-S and -L mRNAs are differentially expressed (Figs. 3 and 4). The differential distribution suggests that DPPX-S and -L catalyze different substrates in spite of their similar potential enzyme action. Because the two forms of DPPX differ in the N-terminal cytoplasmic domain sequences, the differential localization may depend on the sequence difference in the cytoplasmic domain and may, therefore, determine the endogenous substrates on which the enzyme acts. In both yeast and rat DPPs, a short N-terminal cytoplasmic domain has been shown to be important for targeting the protein in the plasma membrane (14, 34).

Some cell surface peptidases have been identified as specific antigens of immune cells. DPPIV has been identified as CD26 expressed in a population of T cells (7, 35), neutral endopeptidase as CD10 expressed by early lymphoid precursors (29, 36), aminopeptidase N as CD13 on committed granulocyte–monocyte progenitors (30, 37), and aminopeptidase A as BP-1/6C3 expressed by early B cells (31, 32). These cell surface antigens have been shown to play important roles in the differentiation of immune cells (31, 32, 35–37), although the molecular mechanism of this phenomenon has not been characterized. By analogy to this phenomenon, either or both DPPXs may be involved in a similar mechanism in transmembrane signaling because of their structural similarity. DPPXs have longer cytoplasmic domains compared with other cloned membrane-bound hydrolases (5, 6, 14, 27–33). The longer cytoplasmic domains of DPPXs may reflect an interaction with some intracellular components. Since the two forms of DPPXs have different cytoplasmic domains produced by an alternative splicing of RNA, the two forms may be involved in different transmembrane signaling systems located in their respective regions. Several proteins have been shown to differ in neuronal and nonneuronal forms by an alternative splicing event (38). In some of the cases, a functional difference in the signal transduction pathway has been suggested between the two forms (39, 40).

Because DPPX-S and -L cDNA clones do not share an identical sequence near the 5′ end, the two forms of DPPX mRNAs are likely to be produced using different promoters. Similar cases of multiple promoters have been reported for several of the vertebrate genes including α-amylase (41), aldose A (42), growth-hormone-releasing hormone (43), and myosin light chain (44, 45) genes. However, except for myosin light chain gene, the proteins encoded by the multiple transcripts are identical since only the 5′ noncoding nucleotide sequences are different from each other (38, 44, 45). In the myosin light chain gene, the two promoters have been shown to determine the internal exon usage that causes the two forms of the protein; however, both forms are expressed in the same tissue (44, 45). Isolation and characterization of the multiple promoters of DPPX gene should give us important information about the tissue- and region-specific expression of the two forms of DPPX.

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