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# Non-conservation of a catalytic residue in a dipeptidyl aminopeptidase IV-related protein encoded by a gene on human chromosome 7

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Previously, we isolated cDNA clones for a new dipeptidyl aminopeptidase IV (DPPIV, EC 3.4.14.5)-related protein (DPPX) from bovine and rat brain libraries (1). Although DPPXs showed the highest amino acid sequence identity (~30%) and similarity (~50%) to cloned mammalian DPPIV (2–4), DPPXs failed to show any dipeptidyl aminopeptidase activity (1). In contrast, DPPIV is a functional serine peptidase that cleaves Xaa-Proline dipeptides from the $NH_2$-terminus of polypeptides (5). Recent molecular characterization of peptidases and lipases has placed DPPIV in a new family in which a serine, an aspartate and a histidine are proposed to form a catalytic triad (3,6). In rat DPPIV, the serine residue of the triad has been confirmed to be a catalytic residue (7). Comparison of amino acid sequences of rat and bovine DPPXs with those of mammalian DPPIVs showed that the first serine residue of the proposed catalytic triad of DPPXs in the two species is not conserved. In the present study, we have isolated human DPPX cDNAs, and studied the molecular evolution of DPPX compared to DPPIV.

A human hippocampus cDNA library (Stratagene) was screened to isolate a human homologue using a fragment of the previously isolated bovine DPPX clone as a probe (1). Plaque-hybridization and clone-isolation were carried out as described previously (1). Inserts of isolated human DPPX cDNA clones were sequenced in both directions by using the dideoxy chain termination method (8). As previously seen in rat and bovine, two distinct forms of DPPX cDNA were identified (Sequences are available from GenBank/EMBL). Predicted proteins (DPPX-S and -L) shared an identical transmembrane domain and a long C-terminal extracellular domain where the catalytic triad is located, but had short and long N-terminal cytoplasmic domains, respectively. Both human DPPX-S and -L showed more than 90% identity in amino acid sequence to previously cloned bovine and rat DPPXs. In human DPPXs, as well as rat and bovine DPPXs, the first serine residue of the proposed catalytic triad was not conserved (Fig. 1). An aspartate was substitued for the serine residue in all three species.

Based on substrate specificity, previous biochemical studies found at least four different enzyme proteins (DPPI through IV) which account for DPP activity in mammalian tissues (5). As observed in rat and bovine DPPXs, expression studies of human DPPX-S and -L cDNAs failed to show any dipeptidyl aminopeptidase activity of DPPX to synthetic peptides which are used to characterize DPPI through IV (N.Y. and K.W., unpublished observation). The result of sequence comparison of DPPX with other functional serine peptidases led us to study a possibility that the lack of enzyme activity of DPPXs is due to the substitution of an aspartate residue for the serine residue in the proposed catalytic triad (Fig. 1). We have introduced mutations into human DPPX-S cDNA to obtain a serine residue at the corresponding position.

Two mutant cDNAs (D650S, K649W-D650S) were prepared using mutagenic oligonucleotides: 5′-GCCGTGTTCGGGAAG <u>TC</u>TTACGTGGCTACCT-3′ and 5′-GTGGCCGTGTT-CGGG<u>TG</u>G<u>TC</u>TTACGGTGGCTACCT-3′, respectively (underline: mutagenic sequences). The translated product of D650S has a single amino acid substitution from original DPPX (from an Asp to a Ser at position 650). The translated product of K649W-D650S has an additional substitution at position 649 (from a Lys to a Trp). Table 1 shows the result of the expression studies of wild type and mutated DPPX-S cDNAs. Membrane fractions of simian COS-7 cells transfected with wild type DPPX-S cDNA did not hydrolyze Gly-Pro-p-nitroanilide above the level of activity of control membrane fractions. Gly-Pro-p-nitroanilide is often used as a substrate for DPPIV since DPPIV is a post-proline cleaving exopeptidase and specifically cleaves Gly-Pro from the $NH_2$-terminus of polypeptides (5). As shown in Table I, transfection of D650S and K649W-D650S retaining the catalytic triad sequence into COS-7 cells produced higher DPPIV activity than that of the control (9.5, 13.4 and 6.4 nmol/min/mg of protein, respectively).

In the present study, mutations were introduced only in DPPX-S cDNAs. However, it is likely that DPPX-Ls with the same mutations would hydrolyze the substrate for DPPIV, because DPPX-S and -L share an identical C-terminal extracellular domain where catalytic sites are located. Transfection of wild type DPPIV cDNA into similar simian cells produced more than

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                 650            730             762
Human DPPX       FGKDYGG........PTADEKI........DESHYFT
Rat DPPX         FGKDYGG........ATADEKI........DESHYFH
Bovine DPPX      FGKDYGG........ATADEKI........DESHYFS

Rat DPPIV        WGWSYGG........GTADDNV........DEDHGIA
Mouse DPPIV      WGWSYGG........GTADDNV........DEDHGIA
Human DPPIV      WGWSYGG........GTADDNV........DEDHGIA
Yeast DPPB       FGWSYGG........GTGDDNV........DSDHSIR
Rat AcH          MGGSHGG........GQEDRRV........KSNHALS
Pig AcH          MGGSHGG........GQEDRRV........KSTHALS
Pig PeP          NGGSNGG........ADHDDRV........KAGHGAG
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**Figure 1.** Comparison of putative catalytic site of previously cloned non-classical serine proteases with the sequence of corresponding areas of DPPXs. Sequences are numbered according to the human DPPX numbering system. DPP, dipeptidyl aminopeptidase; AcH, acyl-amino acid hydrolase; PeP, prolyl endopeptidase. Sequences are taken from human DPPX (this study), rat DPPX (1), bovine DPPX (1), rat DPPIV (2), mouse DPPIV (3), human DPPIV (4), yeast DPPB (22), rat AcH (23), pig AcH (24), and pig PeP (25). The residues of the proposed catalytic Ser-Asn-His triad (3,6) are shown in bold style.

**Table I.** cDNA Mediated Expression of DPPIV activity in COS-7 cells

| Plasmid | Enzyme Activities[a] |
|---|---|
| | (nmol product/min/mg of Protein) |
| K649W-D650S | 13.4 ± 1.3 |
| D650S | 9.5 ± 1.3 |
| Wild type | 6.2 ± 1.4 |
| Control | 6.4 ± 1.6 |

Two mutant cDNAs (K649W-D650S, D650S) were prepared using a Muta-Gene mutagenesis kit (Bio Rad) as described previously (19). A cDNA of a similar size that is unrelated to DPPX cDNA was used as a control. Each cDNA was subcloned into an eukaryotic expression vector, pcDNAI (Invitrogen). The expression vectors (10 mg) carrying wild type, mutants and control cDNAs were transfected into COS-7 cells ($1 \times 10^6$ cells per 100 mm dish) by a calcium phosphate method (20). The transfected cells were incubated for 72 hr in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Then, cells were harvested, and homogenized in 5 mM TrisHCl (pH 7.2) containing 0.25M sucrose and 1mM $MgCl_2$. The pellets obtained after centrifugation at $54{,}000 \times g$ for 30 min were resuspended in 50 mM TrisHCl (pH7.4). The resuspended membrane fractions were subjected to enzyme assay for DPPIV with 1.5 mM Gly-Pro-p-nitroanilide (Sigma) as a substrate according to the previously established method (21). Protein concentration of each sample was determined using BCA protein assay kit (Pierce). [a]Values are Mean ± S.D. of nine individual experiments.

a two hundred-fold increase of enzyme activity compared to the control (7). The lower ratio of the increase of the enzyme activity in the transfection of mutated DPPXs suggests that an active site(s) other than the three residues forming the proposed catalytic triad is required for the expression of fully functional DPPIV activity. This idea is supported by a recent study of site-directed mutagenesis of DPPIV cDNA (7). The finding that K649W-D650S produced more DPPIV activity than D650S also supports this idea.

Human DPPX gene is assigned to chromosome 7 (Table II) by using a BIOSMAP kit (BIOS). According to the manufacturer's protocol, a segment of DPPX gene was amplified using a panel of genomic DNAs from various somatic human×hamster hybrids with a set of primers designed from a deposited sequence at 2452–2471 and 2612–2631 for DPPX-S cDNA. After 30 cycles of amplification by polymerase chain reaction (9), each product was analyzed on an agarose gel. DPPX sequence was amplified only from somatic cell hybrids containing human chromosome 7. The primers did not amplify any sequences from control hamster DNA. In contrast to the DPPX gene, the human DPPIV gene has been mapped to chromosome 2 (10). Different localizations of these genes suggest that DPP genetic loci are not clustered in the mammalian genome. Comparative linkage study on the mouse homologue of human DPPX gene has shown that the mouse gene is linked to markers in the proximal end of chromosome 5 (K.W. and C.A.Kozak, unpublished data). The mouse map location of DPPX gene places it in a region homologous to human chromosome 7q. This supports the result of the present mapping study of human DPPX gene.

In proteolytic enzymes, functionally active amino acid residues are believed to be conserved in evolution (11,12). Significant sequence homology between DPPX and DPPIV suggests that the two genes arise from a common ancestral gene. The lack of a functionally important amino acid in DPPX appears to be an example which would support re-considering the molecular evolution of proteases. It remains possible that endogenous DPPXs hydrolyze an unknown substrate(s). However, this possibility appears to be unlikely because sequences of DPPXs are closely related to those of DPPIVs, and synthetic peptides which are used to characterize all known DPPs were not substrates for DPPXs. Furthermore, DPPXs lack the serine residue forming the proposed catalytic triad. Lack of DPP activity

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**Table II.** Assignment of human DPPX gene to chromosomes in Chinese hamster–human somatic cell hybrids

| Presence of sequence/ Presence of chromosome | Human chromosomes 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Concordant | | | | | | | | | | | | | | | | | | | | | | | | |
| +/+ | 0 | 0 | 0 | 1 | 1 | 1 | 2 | 1 | 0 | 0 | 0 | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 2 | 1 | 2 | 0 | 0 | 1 |
| −/− | 20 | 22 | 19 | 22 | 3 | 20 | 23 | 19 | 20 | 20 | 20 | 20 | 19 | 17 | 20 | 20 | 21 | 19 | 18 | 21 | 18 | 19 | 20 | 20 |
| Discordant | | | | | | | | | | | | | | | | | | | | | | | | |
| +/− | 2 | 2 | 2 | 1 | 0 | 1 | 0 | 1 | 2 | 2 | 2 | 1 | 0 | 1 | 1 | 2 | 2 | 2 | 0 | 1 | 0 | 2 | 2 | 1 |
| −/+ | 3 | 1 | 4 | 1 | 15 | 3 | 0 | 4 | 3 | 3 | 3 | 3 | 4 | 6 | 2 | 2 | 2 | 4 | 5 | 2 | 5 | 4 | 3 | 3 |
| Total Discordant hybrids | 5 | 3 | 6 | 2 | 15 | 4 | 0 | 5 | 5 | 5 | 5 | 4 | 4 | 7 | 3 | 4 | 4 | 6 | 5 | 3 | 5 | 6 | 5 | 4 |
| Total informative hybrids | 25 | 25 | 25 | 25 | 19 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 24 | 24 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| % Discordant | 20 | 12 | 24 | 8 | 79 | 16 | 0 | 20 | 20 | 20 | 20 | 16 | 16 | 28 | 13 | 17 | 16 | 24 | 20 | 12 | 20 | 24 | 20 | 16 |

Hybrids in which a particular chromosome was structurally rearranged or present at less than 10% of the cell population were excluded from the analysis.

in wild type of DPPXs may also raise the possibility that the DPPX cDNAs are derived from the transcripts of a pseudogene (13). However, this possibility is unlikely, because DPPX was originally purified from bovine brain (1) and Southern-blot analysis of human and mouse genomic DNA showed that DPPX gene exists as a single copy (K.W. and C.A.Kozak, unpublished observations).

The predicted membrane topology of DPPX is similar to that of multi-functional transmembrane peptidases including DPPIV (14). It has been well documented that DPPIV is involved in many physiological processes such as cell-matrix adhesion in hepatocytes (15), transmembrane signaling in immune cells serving as a cell-surface marker, CD26 (16), and peptide absorption in apical brush border in gut and kidney (17). In hepatocytes, the interaction of DPPIV with the extracellular matrix does not involve the catalytic site of DPPIV (15). Since that reverse transcription-polymerase chain reaction analysis showed that DPPX mRNAs are expressed predominantly in mammalian brain (1) and that DPPX is closely related to DPPIV, it is likely that endogenous DPPX is involved in important physiological processes of brain function. The comparative linkage study on the mouse homologue of human DPPX gene suggested the potential linkage of mouse DPPX gene to *reeler* (K.W. and C.A. Kozak, unpublished observations). The locus is known to be a recessive neurological marker characterized by difficulties in locomotion with pathological defects caused by abnormal neuronal cell migration (18). We are investigating the possibility that DPPX gene is involved in the pathogenesis of *reeler*.

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