Aspartyl Aminopeptidase, Encoded by an Evolutionarily Conserved Syntenic Gene, Is Colocalized with Its Cluster in Secretory Granules of Pancreatic Islet Cells

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Aspartyl aminopeptidase (DAP), encoded by the DNPEP gene, is believed to be a cytosolic protein with high enzymatic activity in the neuroendocrine tissues. Bioinformatic analysis revealed that the genomic segment spanning the DNPEP gene is evolutionarily conserved from Caenorhabditis elegans to humans. In the present study, we sought to determine whether the expression of DAP is associated with its clustered genes when expressed in pancreatic islet cells. Using anti-DAP specific antibody in immunofluorescent stainings, we found that DAP was specifically expressed in islet alpha cells but not in exocrine acinar cells. Moreover, using electron microscopy, we found that DAP was associated with a lysosomal-like structure and secretory granules, suggesting that it plays an important role in post-translational processing and the secretion of hormones in islet cells. The identification and characterization of DNPEP syntenic genes confirm that conserved clustered genes can preferentially be expressed in the same signaling pathway.

Key words: aspartyl aminopeptidase; pancreatic islets; secretory granules; comparative genomics; synteny

Many homologous genes are clustered in genomes and evolutionarily conserved in syntenic segments cross-species,1,2 including immunoglobulin genes3 and chemosensory receptor genes.4 As more genomes are sequenced, more syntenic clustered genes will be identified through comparative genomics, which will dramatically enhance understanding of the significance of functional genomes in the physiology and pathophysiology of life.

Since Type 1 diabetes is associated with islet cell dysfunction, understanding how hormones such as insulin and glucagon are produced and regulated for secretion in the islets of the pancreas is important for the prevention and treatment of Type 1 diabetes. Thus, bioinformatic analysis and subsequent localization studies of clustered genes related to islet function may provide clues for further studies of their roles in glucose metabolism. PTPRN (Protein Tyrosine Phosphatase Receptor type N, gene ID number 5798) is a secretory granule membrane protein in islet cells and a major autoantigen in Type 1 diabetes.5,6 It is composed of a cytoplasmic region (a.a. 600–979) and a luminal region (a.a. 1–576), which has significant homology at a.a. 1–200 with RESP18.7 Ptprn-deficient mice showed reduced insulin secretion from isolated islets and glucose intolerance.8 PTPRN is evolutionarily conserved from C. elegans to humans9 and a mutation of the PTPRN orthologous gene in C. elegans also impaired the insulin-like pathway.10

Sequence analysis and chromosome mapping revealed that PTPRN was clustered with RESP18 at human chromosome 2q35.11 Further investigation confirmed that RESP18 also encoded a secretory granule protein in islet cells and formed a syntenic block with PTPRN across the mammalian species examined,12 but not in invertebrates. DNPEP, another neighbor gene of PTPRN, encodes aspartyl aminopeptidase (DAP, EC 3.4.11.21), a mammalian homolog of the M18 family in yeast, which is believed to be a cytosolic protein with high enzymatic activity in neuroendocrine tissues. Brain aspartyl aminopeptidase, which converts angiotensin I to angiotensin 2–10, was identified as a possible target for antihypertensive therapy.13 In this present study, we found that DNPEP was mapped to the same block as PTPRN, and that DAP was co-localized with glucagon in secretory granules in pancreatic islet cells, suggesting that genes from this syntenic block also function coordinately.

Materials and Methods

Bioinformatic analysis. The entire sequence and predicted genes of human DNPEP-containing BAC clone (GenBank acc. no. AC053503) were analyzed by BLAST searching the National Center for Biotechnology Information databases (http://ncbi.nlm.nih.gov/BLAST) with BLAST algorithms.12 The adjacent genes of DNPEP, including PTPRN and RESP18, were investigated to determine whether the orthologs could be mapped to the vicinity of DNPEP locus in the genome of other species. Domains and motifs were predicted with SMART (http://smart.embl-heidelberg.de).

The loci conservation of syntenic genes shared between C. elegans and humans was determined by database search (NCBI, Ensembl, Xenbase G-Browse, Ensembl Metazoa, and Wormbase) the genome assemblies of 12 species, including seven mammals (human, Homo sapiens; chimpanzee, Pan troglodytes; monkey, Macaca mulatta; cow,
Box taurus; dog, Canis familiaris; rat, Rattus norvegicus; mouse, Mus musculus; one bird (chicken, Gallus gallus), one amphibian (western clawed frog, Xenopus tropicalis), one fish (zebra fish, Danio rerio), two insects (fruit fly, Drosophila melanogaster; mosquitoes, Anopheles gambiae), and one worm (nematode, Caenorhabditis elegans).

Antibody and microscopy. Antibody to DAP was the gift of Dr. S. Wilk. Antibody to RESP18 was the gift of Dr. M. Schiller. Antibody to PTPRN was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-insulin, anti-glucagon, and anti-somatostatin mouse monoclonal antibodies were from Sigma (St. Louis, MO). Parailin embedded slides of mouse pancreas tissue were prepared, and immunofluorescent stains were performed by standard methods. Microscopic analysis was done with a Zeiss Axioshot microscope (Columbus, OH).

The Allan Brain Atlas. The Allan Brain Atlas (ABA) (http://www.brain-map.org) supports gene-specific expression analysis in the mouse brain by providing an automated high throughput in situ hybridization internet platform. In situ hybridization data for the expression of each gene in adults were extracted from the ABA, a publicly accessible online gene expression digital library widely cited in the literature. All ABA image-based, in situ hybridization data are spatially registered to the common anatomic framework of the ARA with a standard coordinate system and hierarchical ontology. The Anatomic Gene Expression Atlas (AGEA) in principle enables users to view a comprehensive list of genes expressed in any given three-dimensional voxel of the mouse brain atlas by using the Gene Finder application. Expression levels in different brain regions were downloaded as xml files and processed with Microsoft Excel 2007. Sagittal planes of the spinal cord were taken at the cervical spinal level (C1) for further analysis of Dnep, Resp18, and Ptprn expression data.

Immunoelectron microscopy. Mouse pancreatic islets were isolated, fixed, and embedded by standard methods. Immunogold Electron Microscopy was performed as follows: Briefly, the sections were exposed to blocking solution containing 2% BSA, 2% normal goat serum, and 0.1% fish gelatin in PBS containing 0.05% Tween-20 (PBST) for 60 min, and incubated with anti-DAP polyclonal antibody (1/10 dilution) and anti-glucagon monoclonal mouse antibody (1/50 dilution). The primary antibodies were detected with 5-nm diameter colloidal gold particles conjugated to goat-anti-rabbit IgG and 10-nm diameter colloidal gold particles conjugated to goat-anti-mouse IgG respectively (Ted Pella, Redding, CA).

Protein fractionation. The synaptosome, which contains vesicles, was enriched as previously described. Briefly, the whole mouse brain was homogenized in buffer (0.32 M sucrose, 20 mM HEPES, pH 7.4, with protease inhibitor cocktail, Sigma) and centrifuged at 1,000 g for 10 min to pellet the membrane fragments and nuclei. The supernatant was then centrifuged at 17,000 g for 15 min to obtain pellet-containing synaptosomes contaminated with mitochondria and microsomes. This crude synaptosomal fraction was further purified using a discontinuous sucrose density gradient consisting of a 0.8-M sucrose layer on the top and a 1.2-M sucrose layer on the bottom. The pure synaptosomal fraction was obtained from the interface of 0.8-M and 1.2-M sucrose after 90 min of centrifugation at 54,000 g. The lysosome- or nuclei-containing fraction was then enriched with other appropriate reagents (Instructions 89839 and 89841 respectively, Pierce, Rockford, IL).

DAP enzymatic activities. Membrane-bound DAP activity was measured as previously described in buffer of 50 Tris–HCI (pH 7.4), 1 mM MnCl₂, and 0.125 mM aspartyl-beta-NA. Relative fluorescence was converted into pmoles of product using a standard curve, constructed with increasing concentrations of beta-naphthylamine. The results were recorded as units of aminopeptidase per mg of protein of analyzed tissue. One unit of aminopeptidase activity was the amount of enzyme that hydrolyzed 1 pmol of aminoyl-beta-naphthylamine per min. Protein concentrations were measured by Micro BCA Protein Assay (Pierce). Data were evaluated by one-way analysis of variance (ANOVA), followed by the DMS test for comparisons among more than two groups.

Fig. 1. Syntenic Segment Encompassing PTPRN, RESP18, and DNPEP. Each box represents individual genes. Synteny conservation is highlighted by the same color between various species. The synteny structures in all five species (chimpanzee, monkey, cow, dog, and rat) between human and mouse are similar to that in human and mouse, and hence are skipped, as indicated by broken arrows. The orthologous genes in non-mammal vertebrates, including chicken, Xenopus, and zebrafish, are also clustered. The orthologous genes in the invertebrate C. elegans were intervened by 17 bystander genes (not displayed). The name of each gene is given on the top row. The names of species and chromosome numbers (in parentheses) are given on the left.

Results

DNPEP was tandemly arranged with PTPRN in a conserved syntenic region

That PTPRN was clustered with RESP18 across mammalian species and matched the sequence at the first 200 amino acids in the luminal region, and was expressed in the same subcellular location in the islet cells of the pancreas, prompted us to hypothesize that the conserved region of PTPRN has additional genes sharing properties, including a similar expression pattern. Hence, we analyzed the 163-kb PTPRN-containing BAC clone (RP11–74C8) on human chromosome 2q35. We found that orthologs of PTPRN, RESP18, and DNPEP, clustered in human genome, were also present in the same order on mouse chromosome 1. A similar syntenic segment (PTPRN-RESP18-DNPEP in order) was found in all seven of the mammalian species we examined, including Homo sapiens (chromosome 2q35), Pan troglodytes (Chr. 2b), Macaca mulatta (Chr. 12), Bos taurus (Chr. 2), Canis lupus familiaris (Chr. 37), Rattus norvegicus (Chr. 9q33), and Mus musculus (Chr. 1) (Fig. 1). To determine whether the syntenic block was evolutionarily conserved in non-mammal animals, we searched and analyzed genomes in further species, including Danio rerio, Drosophila melanogaster, and C. elegans. The ortholog of RESP18 was not found in any of the non-mammal species we examined. The ortholog of DNPEP was not found in the insect species of Drosophila melanogaster or Anopheles gambiae. However, ortholog genes of PTPRN and DNPEP were found in the other four examined species, including Gallus gallus (Chr. 7), Xenopus tropicalis (Chr. location...
unknown), Danio rerio (Chr. 6), and C. elegans (Chr. III), suggesting that PTPRN-DNPEP is a several-million-year-old syntenic segment. We also found that the cluster was duplicated in Danio rerio (Chr. 9 and Chr. 6), but the Dnpep gene on Chr. 9 of Danio rerio was missing. In C. elegans, there are 17 other genes in the region (less than 100 kb) between Ptprn (also known as ida-1) and Dnpep (e.g., F01F1.9). No identical expression patterns were identified in the 17 genes according to the Worm Database. Moreover, none of these 17 genes was syntenically mapped to the PTPRN-DNPEP-containing regions in other species.

DAP was expressed in the pancreatic islets
Since the 5’-terminal flanking region of DNPEP showed considerable similarity to those of PTPRN and RESP18 (data not shown), it appeared that the expression of these genes is regulated by common transcription factors for their specific expression in islet cells. As shown in Fig. 2A and B, PTPRN and RESP18 were both expressed in islet cells, with a stronger signal in the peripheral region. Using DAP-specific rabbit polyclonal antibody, we performed immunofluorescent staining of mouse pancreas sections. Our results indicated that DAP was also localized to the peripheral cells of the islets (Fig. 2C). This specific pattern suggest that DAP was expressed in glucagon-positive alpha cells or somatostatin-positive delta cells. Further analysis confirmed that the DAP fluorescent signal was completely co-localized with glucagon, as detected by anti-glucagon monoclonal antibody (Fig. 2D and E), but not co-localized with insulin beta cells or somatostatin delta cells (not shown).

Dnpep was expressed in neuronal tissues in a pattern similar to Ptprn and Resp18
To determine whether Dnpep expression in neuronal tissues is similar to Ptprn and Resp18, we analyzed the area where messenger RNAs that encode DAP, RESP18, and PTPRN proteins are expressed in adult (56 d) mouse brain tissues, using the Allen Brain Atlas (ABA) data sets. The probes used for in situ hybridization were 781 bp of mouse Dnpep (nt 1058–1819, GenBank acc. no. NM_001110831), 591 bp of mouse Resp18 (nt 60–650, GenBank acc. no. NM_009049), and 713 bp of mouse Ptprn (nt 1947–2659, GenBank acc. no. NM_008985). The identities and similarities among these probes were low (<40–45%), with no significance. However, the expression patterns of Dnpep, Resp18, and Ptprn were very similar, and were present in various regions of brain (Fig. 3). Detailed analysis showed that higher expression levels of Dnpep, Resp18, and Ptprn were in the midbrain, pons, thalamus, main olfactory bulb, and hippocampus regions. Lower expression levels of these genes were found in the subthalamic nucleus, hippocampal region, cerebral cortex, caudate putamen, and cerebellum, as indicated in the Fig. 3.

Dnpep, Resp18, and Ptprn were expressed in similar patterns in all 20 segments of the spinal cord, from the cervical to the lumbar spine. As Fig. 4 illustrates, the mRNAs of the three genes were similarly distributed in the uppermost cervical segment of the spinal cord of the adult mice, located mainly in neuron-rich regions of gray matter.

DAP was found in secretory granules
To examine subcellular localization of DAP in islet cells further, we conducted immunoelectron microscopy analysis with anti-DAP antibody. Electron micrographs revealed that DAP was associated with lysosomal-like structures (Fig. 5A) as well as secretory granules in the islet alpha cells (Fig. 5B). Quantitative analysis of DAP particles per square micrometer in 20 electron microscopic images revealed that there were 84 ± 13 particles...
around the lysosome and $32 \pm 9$ in secretory granules compared to $9 \pm 3$ particles in the cytoplasm (mean ± SD, $p < 0.01$). By double immunolabeling with rabbit antibody to DAP (5-nm colloidal gold particles conjugated to goat anti-rabbit antibody) and mouse antibody to glucagon (10-nm of colloidal gold conjugated with goat anti-mouse antibody), we found that DAP was co-localized with glucagon in islet alpha cells (Fig. 5C).

**DAP enzymatic activities detected in the mouse brain**

Whether DAP aminopeptidase activity in the cells was consistent with the subcellular distribution found in the electron microscopic analysis, we measured DAP enzymatic activities in fractionated mouse brain tissues. Since DAP was found in secretory granules in the islet cells, we assumed that DAP might be co-localized with vesicles in the neuronal cells of the brain. Synaptosomes that contain secretory vesicles and mitochondria of presynaptic terminal and postsynaptic membranes were prepared from brain tissues by classical subcellular fractionation techniques. Lysosome-, microsome-, and nuclei-enriched fractions were also prepared for enzymatic activity analysis. Our data indicated that the highest activities of DAP (-400 units) were detected in the synaptosomal and the lysosomal fractions (Fig. 6). Although our electron microscopic analysis did not show the presence of DAP in ER and Golgi or nuclei, lower activities were also found in the fractions of microsome (-142 units) and nuclei (-57 units) (Fig. 6). It cannot be excluded, however, that the lower activities were to be attributed to small amounts of heterogenous fractions of cytosol proteins that contained the DAP protein.

**Discussion**

In the present study, we found that *DNPEP* expression was similar to two other tandemly arranged genes, *PTPRN* and *RESP18*, in the same evolutionally conserved syntenic block. We also found that DAP was
expressed in mouse and human islet alpha cells and was subcellularly associated with secretory granules and lysosomal-like compartments. DAP enzymatic activity assay indicated that the highest activities of DAP was in the synaptosomal- and lysosomal-enriched cellular fractions of the brain tissues. These findings confirm that genes located in the same syntenic block can also share similar expression patterns and even related biological functions.

Previous phylogenetic analysis indicated that PTPRN and RESP18 form a conserved syntenic block in mammalian but not in non-mammalian species. Both RESP18 and PTPRN were expressed in secretory granules of the same islet cells and were transcriptionally upregulated upon high glucose stimulation or hyperglycemia. In this study, we determined that DNPEP was physically linked with PTPRN in the genomes of both humans and C. elegans, suggesting a strong syntenic conservation of the block over the evolutionary spectrum of bilaterian animals. Two PTPRN paralog genes (in chromosome 6 and 9 respectively) were identified after whole-genome duplication in the zebrafish. But one DNPEP paralog in chromosome 9 was missing. In fact, a loss of paralogs or ohnologs in zebrafish was frequently observed. This might play a role in establishing lineage divergence and in the origin of developmental innovations. DNPEP is an evolutionarily conserved gene found from C. elegans to humans. DNPEP was missing in the insects (Drosophila melanogaster and Anopheles gambiae), although PTPRN was present in both insects. Also, it is not uncommon that all other functionally unrelated bystander genes between PTPRN and DNPEP, as in the case of C. elegans, are not clustered with PTPRN or DNPEP and lost in other species after millions of years of evolutionary divergence and selection. Considering that common ancestors of C. elegans and zebrafish in vertebrates diverged 450–500 million years, we hypothesize that conserved genes within the same syntenic block keep similar tissue specific expression patterns in neuroendocrine tissues, including islets and neuronal tissues in the brain and the spinal cord, and hence are also involved in similar biological functions. Using DAP-specific antibody in immunostaining and electron microscopy, we found that DAP was indeed expressed in the hormone secretory granules in islet cells, like its neighbors PTPRN and RESP18.

In situ hybridization analysis showed very similar expression patterns for Dnpep, Resp18, and Ptprn in both the brain and the spinal cord. Since genes expressed in the same tissue and cellular localization can have similar regulatory elements in their highly conserved noncoding elements (HCNEs), identification of such genomic regulatory blocks (GRBs) with specific expression patterns in pancreatic endocrine cells may provide clues to promote further investigation into the corresponding biological functions of these genes.

DAP is believed to be a cytosolic enzyme associated with the lysosome in mammals and the vacuoles in yeast. The mammalian DAP is preferentially expressed in neurons and neuroendocrine tissues. High activity of DAP was found in the testis, the kidney, and the myelinic and synaptosomal fractions of brain tissues. PTPRN and RESP18 are also associated with secretory granules and granule-enriched fractions in the

Fig. 5. Subcellular Distribution of DAP in Islet Alpha Cells.

Thin sections of mouse pancreatic islet tissue were used in immunoelectron microscopy analysis. Anti-DAP rabbit antibody was detected with goat anti-rabbit IgG antibody conjugated to colloidal gold. (A) DAP particles were abundant around the lysosomal-like structure (10 nm colloidal gold) and were also detected in the luminal region of the secretory granules (B, 5-nm colloidal gold), as indicated by arrows. DAP particles were not detected in the mitochondria or nuclei. (C) DAP (5 nm colloidal gold particles, black arrows) was co-localized with glucagon (detected with 10-nm colloidal gold particles conjugated to goat anti-mouse antibody, white arrows) in DCVs. Scale bar, 100 nm.

Fig. 6. Subcellular Distribution of DAP Enzymatic Activities in the Mouse Brain.

Values are expressed as units of aminopeptidase per mg protein, from three independent experiments (mean ± SEM).
brain and endocrine cells.\(^{6,28}\) Moreover, DAP was reported to convert angiotensin I to angiotensin II-10,29) and also to degrade angiotensin II to angiotensin III,\(^{23}\) suggesting that DAP plays a major role in blood pressure (see a recent review\(^{11}\)). PTPRN was recently found in the kidney as well. Knockout of the Ptprn gene in mice resulted in a marked reduction of renin-angiotensin levels in plasma,\(^{30}\) which suggests that DAP and PTPRN play similar roles in regulating blood pressure in the kidney. The RESPI8 protein was also found in the kidney tissue by Western blot.\(^{31}\) Whether DAP and PTPRN play similar roles in regulating blood catabolism of hormones and peptides in the secretory granules and vesicles might contribute to understanding of this phenomena.

In conclusion, through genomic localization, tissue specific expression, subcellular localization, and enzyme activity studies, we found that DAP and PTPRN are co-localized in the secretory granules of pancreatic islet cells, signifying that DAP may play an important role in the post-translational processing and secretion of hormones. Further studies are required to determine the functional relationships of DNPEP with the other members of the same syntenic block.

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