A containing 0.05% Triton X-100.

Western Blotting—Purified DPP IV was electrophoresed on a 7.5% SDS-PAGE gel and electrotransferred to PVDF membranes. After the electroblotting, the membranes were treated with rabbit IgG anti-rat kidney antibody (10 μg/ml) or rabbit IgG anti-peptide antibody (25 μg/ml). HRPO-conjugated Protein A was used as a second antibody. The enhanced chemiluminescence (ECL) system (Amersham) was used for detection. The membranes were subsequently exposed to Hyperfilm-^{3}H autoradiography film and processed.

N-Glycanase Treatment—An equal volume of 40 mM NaH_2PO_4-Na_2HPO_4 (pH 8.0) was added to purified DPP IV (10 μl, 1.5 μg protein), and the mixture was boiled in the presence of SDS and β-mercaptoethanol for 5 min to denature the protein. Then 7.5% Nonidet P-40 (5 μl) and 250 U/ml recombinant *N*-glycanase (2 μl) were added, and the mixture was incubated for 21 h at 37°C.

DPP IV Binding to Adenosine Deaminase—Samples of serum DPP IV (72.5 mU) and kidney DPP IV (52 mU) were incubated for 30 min at 37°C in the presence or absence of calf intestinal ADA (ADA-S, 1 μg) in a total volume of 20 μl. The reaction mixtures were run on a 4-20% non-SDS-PAGE gel. Proteins on the gel were detected by Coomassie Brilliant Blue staining and by Western blotting using a specific rabbit IgG anti-calf ADA antibody (10 μg/ml). Alkaline phosphatase conjugated anti-rabbit IgG (H+L) was used as a second antibody and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Wako Pure Chemical Industries) was used as substrate.

RESULTS AND DISCUSSION

Purification of Human Serum DPP IV—Human serum DPP IV was isolated by sequential purification using ion exchange chromatography (DEAE-Cellulose DE-52 and DEAE-Cellulofine A-800), gel filtration on a Sephacryl S-300 HR column, and hydrophobic chromatography on a butyl-Toyopearl 650S column. The purification steps for DPP IV are summarized in Table I. Serum DPP IV was purified 14,400-fold with a 25% yield over serum. Its specific activity was 19.1 μmol/min/mg protein with Gly-Pro-pNA as the substrate. The purified enzyme migrated as a single band on SDS-PAGE under reducing conditions (Fig. 1). The molecular mass of the enzyme was estimated to be 110 kDa, similar to that of the human membrane-bound kidney DPP IV. The purified enzyme also showed the same molecular weight band under non-reducing conditions (data not shown). We have previously reported that our anti-rat kidney DPP IV antibody recognizes a 60-kDa fragment of rat kidney DPP IV cut off at the 281st residue from the N-terminus (*34*). DPP IVs from serum and kidney showed cross-reactivity to the anti-rat kidney DPP IV antibody in Western blotting, and their molecular sizes were identical (data not shown).

Comparison of Enzymatic Properties of Kidney and Serum DPP IVs—The relative rates of hydrolysis of various pNA substrates by the purified enzyme preparations are shown in Table II. Kidney and serum DPP IVs showed almost the same substrates specificities. To characterize the specificities and action of the enzymes further, the hydrolysis of some biologically active peptides was investigated. Substance P and β-casomorphine-5 were used. Both peptides are known to contain Pro residues at the second and the fourth positions from the N-termini, and the N-terminal dipeptide and subsequent dipeptide from substance P and β-casomorphine-5 are hydrolyzed by DPP IV (*35*). No difference in the hydrolysis of these compounds was found between the two DPP IVs (data not shown).

Kidney DPP IV is a serine protease and an aminopeptidase which can be inhibited by DFP (*25*) and diprotin-A (*36*), respectively. We examined the effects of DFP and diprotin-A on DPP IVs from kidney and serum. Both DPP IVs were inhibited by DFP and diprotin-A in the same manner. ID_{50} of DFP for kidney and serum DPP IVs was 0.55 and 0.50 mM, respectively, and ID_{50} of diprotin-A for kidney and serum DPP IVs was the same (20 μM). Therefore, we can confirm that serum DPP IV is a serine proteinase.

N-Glycanase Treatment of DPP IVs—DPP IV is regularly present as a non-covalently linked homodimeric glycoprotein, consisting of two *N*-glycosylated subunits of 110 kDa (*8*). So we investigated the pattern of glycosylation of the membrane-bound and serum forms. As shown in Fig. 2, the deglycosylated backbone of serum DPP IV is slightly smaller than that of kidney DPP IV. This result demon-

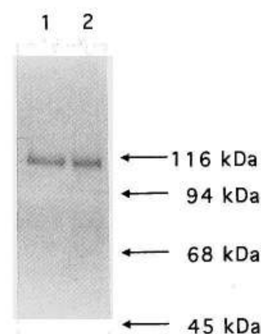

Fig. 1. **SDS-PAGE of DPP IVs from human normal serum and kidney.** Samples were subjected to SDS-PAGE (7.5% gel) under reducing conditions, followed by protein staining (Coomassie Brilliant Blue R-250). Marker proteins used are β-galactosidase (116 kDa), phosphorylase *a* (94 kDa), bovine serum albumin (68 kDa), and ovalbumin (45 kDa). Lane 1: serum; lane 2: kidney.

TABLE I. **Purification of DPP IV from normal human serum.**

Step	Total protein (mg)	Total activity[a] (units)	Specific activity (mU/mg protein)	Yield (%)	Purification (-fold)
Human serum	12,600	16.8	1.33	100	1
DEAE-Cellulose DE-52	2,490	8.98	3.61	53.5	2.71
DEAE-Cellulofine A-800	1,560	8.68	5.56	51.7	4.18
Sephacryl S-300 HR	102	7.05	68.8	42.0	52.0
Butyl-Toyopearl 650S	0.22	4.20	19,100	25.0	14,400

[a]One unit (U) is defined as the amount of enzyme cleaving 1 μmol of Gly-Pro-pNA/min.

strates that there is a difference in the primary structure of the DPP IVs.

Comparison of the N-Terminal Amino Acid Sequences—To investigate the primary structure of DPP IVs, the N-terminal amino acid sequences of membrane-bound kidney and serum DPP IVs were determined and compared with the amino acid sequence predicted from the cDNA nucleotide sequence of human liver DPP IV (*8*) (Fig. 3). The entire amino acid sequence of human kidney DPP IV including the transmembrane site has not yet been reported. However, we revealed that the N-terminal sequence of human kidney membrane-bound DPP IV was completely identical to the predicted structure. On the other hand, the N-terminal sequence of serum DPP IV was identified in a sequence starting at the 39th position, serine, from the N-terminus. These results suggest that membrane-bound DPP IV loses its transmembrane domain upon release into the serum.

To confirm the loss of the transmembrane domain, the cross-reactivity of serum DPP IV with an anti-peptide antibody against the N-terminus of kidney DPP IV was examined. A peptide fragment consisting of 16 amino acid residues (MKTPWKVLLGLLGAAA) from the N-terminus of kidney DPP IV was synthesized using the Fmoc-polyamide solid-phase method, and a polyclonal antibody was raised in rabbit. Kidney membrane-bound DPP IV cross-reacted with the anti-peptide antibody, but serum DPP IV did not (Fig. 4). This result supports the N-terminal amino acid sequence analysis of serum DPP IV as lacking the transmembrane domain.

Our previous studies have shown that APN and CAP in sera from pregnant women are differently processed derivatives of membrane-bound APN and CAP, respectively (*37, 38*). Furthermore, our recent result indicates that APN in normal human serum is a differently processed derivative from the one in maternal serum (*39*). DPP IV, like APN and CAP, is a type II integral membrane aminopeptidase. We can, therefore, propose with certainty that the type II integral membrane aminopeptidases can be released from the membrane to the blood circulation by differential limited proteolysis, though the mechanism of release remains unclear.

While this study was in progress, Duke-Cohan *et al.* reported that a novel form of DPP IV was found in human serum that was not a cleavage product of CD26/DPP IV (*40*). Their DPP IV existed as a monomer of 175 kDa and the N-terminal sequences of its tryptic digest have no homology with CD26/DPP IV. They also reported finding a novel activated T cell antigen (DPPT-L), and that the 175-kDa DPP IV is similar to DPPT-L released from activated T cells (*41*). We think the inconsistency between their findings and our data might have resulted from their missing the real serum DPP IV during their purification processes. Our conclusion indicates that serum DPP IV is a cleavage product of 110-kDa membrane-bound DPP IV. Furthermore, it seems that what Duke-Cohan *et al.* call 175-kDa DPP IV is quite different from real serum DPP IV

TABLE II. **Comparison of the substrate specifity of DPP IVs from kidney and serum.**

Substrate	Relative activity (%)	
	Kidney	Serum
Gly-Pro-pNA	100	100
Ala-Pro-pNA	66.4	69.1
Val-Ala-pNA	16.9	20.5
Ala-Ala-pNA	14.2	11.5
Z-Gly-Pro-pNA	0	0
Pro-pNA	0	0

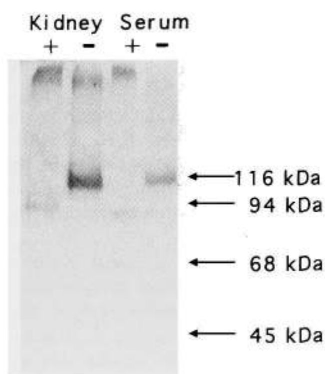

Fig. 2. ***N*-Glycanase digestion of DPP IVs from kidney and serum.** The proteins were incubated overnight at 37°C in the presence (+) or absence (−) of *N*-glycanase as described under "MATERIALS AND METHODS." The digested proteins were subjected to SDS-PAGE (7.5% gel), followed by Western blotting with a rabbit anti-rat kidney DPP IV antibody. Marker proteins used are β-galactosidase (116 kDa), phosphorylase *a* (94 kDa), bovine serum albumin (68 kDa), and ovalbumin (45 kDa).

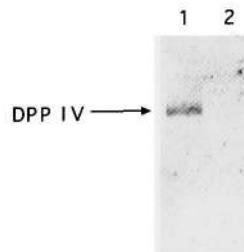

Fig. 4. **Western blotting of DPP IVs from kidney and serum with a rabbit anti-peptide antibody.** Samples were subjected to SDS-PAGE (7.5% gel), followed by Western blotting with a rabbit anti-peptide antibody. Lane 1: kidney; lane 2: serum.

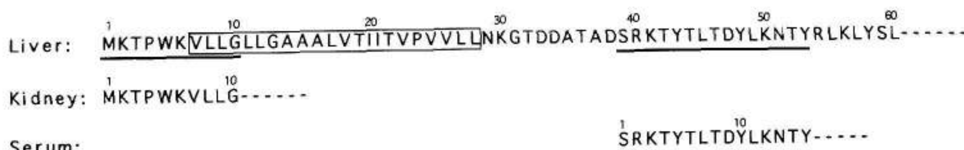

Fig. 3. **Sequence comparison of the human membrane-bound kidney and serum DPP IVs with liver DPP IV.** Sequences of the human liver (*8*), kidney, and serum DPP IVs are aligned: —, identical amino acids; numbers, amino acid residue position. The transmembrane domain is boxed.

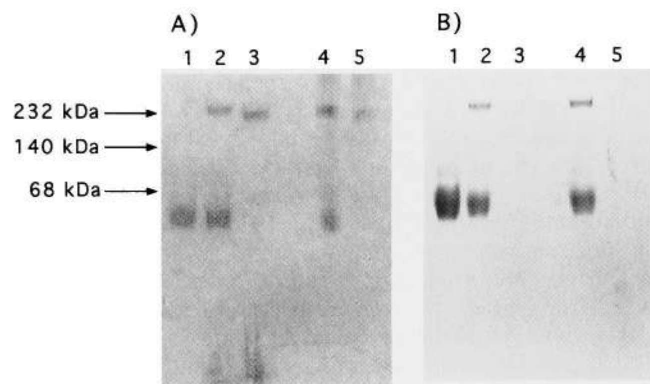

Fig. 5. **Binding of serum and kidney DPP IVs to ADA.** Samples were subjected to non-SDS-PAGE (4-20% gel), followed by Coomassie Brilliant Blue R-250 staining (A) and by Western blotting with a rabbit anti-ADA antibody (B). Lane 1: ADA only; lane 2: binding of serum DPP IV to ADA; lane 3: serum DPP IV only; lane 4: binding of kidney DPP IV to ADA; lane 5: kidney DPP IV only. Marker proteins used are catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (68 kDa).

and may be DPPT-L.

DPP IV Binding to Adenosine Deaminase—Human ADA exists in three forms: a low molecular weight type 1 (ADA-S, 40 kDa); a high molecular weight type 1 (ADA-L, 280 kDa); and an intermediate molecular weight type 2 (ADA-2, 110 kDa) (*42*). ADA-S predominates in almost all intracellular tissues and membranes, and is convertible to ADA-L, which is a complex of ADA-S (2 molecules) and DPP IV (1 molecule). On the other hand, ADA-2 is predominantly found in the serum and its properties have not yet been clarified. As shown in Fig. 5A, serum DPP IV (lane 3), like kidney DPP IV (lane 5), could exist as a dimer in non-SDS-PAGE gel. Upon incubation with ADA-S, the 220-kDa band of both serum and kidney DPP IVs disappeared and a new band with higher molecular weight appeared (lanes 2 and 4). Furthermore, in both serum and kidney DPP IVs, Western blotting using a rabbit IgG anti-ADA antibody showed the translocation of ADA from low molecular weight ADA (ADA-S) to the high molecular weight complex of DPP IV and ADA (ADA-L) (Fig. 5B). These results suggest that ADA-S can associate with both serum and membrane-bound DPP IV. Recently, some groups have reported that ADA-S interacts with the membrane form of DPP IV (*21, 43*), but Duke-Cohan *et al.* reported that DPP IV in human serum did not bind to ADA-S (*40*). From the point of view outlined above, we may question the sample which Duke-Cohan *et al.* used. Our result then suggests that DPP IV dose not need to be fixed on the plasma membrane in order to bind to ADA.

At present, we do not know the mechanism of the release from the membrane to serum, but it is worthwhile to clarify it and to investigate the correlation of serum DPP IV with immune status.

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