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Identification of the Active Site Residues in Dipeptidyl Peptidase IV by Affinity Labeling and Site-Directed Mutagenesis†

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ABSTRACT: The active site of dipeptidyl peptidase IV (DPPIV) was examined by chemical modification and site-directed mutagenesis. Purified DPPIV was covalently modified with [^{3}H]diisopropyl fluorophosphate (DFP). The radiolabeled DPPIV was digested with lysyl endopeptidase, and the peptides were separated by high-performance liquid chromatography. A single ^{3}H-containing peptide was obtained and analyzed for amino acid sequence and radioactivity distribution. A comparison of the determined sequence with the predicted primary structure of DPPIV [Ogata, S., Misumi, Y., & Ikehara, Y. (1989) *J. Biol. Chem. 264*, 3596-3601] revealed that [^{3}H]DFP was bound to Ser^{631} within the sequence Gly^{629}-Trp-Ser-Tyr-Gly^{633}, which corresponds to the consensus sequence Gly-X-Ser-X-Gly proposed for serine proteases. To further identify the essential residues in the active-site sequence, we modified the DPPIV cDNA by site-directed mutagenesis to encode its variants. Expression of the mutagenized cDNAs in COS-1 cells demonstrated that any single substitution of Gly^{629}, Ser^{631}, or Gly^{633} with other residues resulted in the complete loss of the enzyme activity and DFP binding. Although substitution of Trp^{630} → Glu or Tyr^{632} → Phe caused no effect on the enzyme activity, that of Tyr^{632} → Leu or Gly abolished the activity. These results indicate that the sequence Gly-X-Ser-(Tyr)-Gly is essential for the expression of the DPPIV activity.

Dipeptidase peptidase IV (DPPIV)[1] is a unique enzyme among dipeptidyl aminopeptidases. DPPIV is an ectoenzyme, in contrast to lysosomal and cytosolic localization of other DPPs, and it cleaves X-proline dipeptides from the NH_2 termini of peptides (Hopsu-Havu & Glenner, 1966; McDonald & Schwabe, 1977; Macnair & Kenny, 1979). Despite ex-

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[1] Abbreviations: DPPIV, dipeptidyl peptidase IV; DFP, diisopropyl fluorophosphate; HPLC, high-performance liquid chromatograpy; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; *N*-glycanase, peptide *N*-glycosidase F.

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tensive studies on DPPIV, its physiological roles have not been fully revealed. High levels of the DPPIV activity in the brush border of small intestine and kidney proximal tubules suggest that DPPIV participates in the metabolism and uptake of proline-containing peptides in these tissues (Miyamoto et al., 1987; Tiruppathi et al., 1990). On the basis of its unique specificity toward proline, DPPIV has also been postulated to be important in the maturation and degradation of biologically active peptides such as substance P and melittin (Kreil et al., 1980; Puschell et al., 1982; Mentlein, 1988). The purified enzyme is found to be dimeric, comprising two identical subunits of 110–130 kDa which are variable depending on the species and tissue, possibly due to the extent of glycosylation (Kenny et al., 1976; Elovson, 1980; Yamashita et al., 1988).

The complete inhibition of DPPIV activity with diisopropyl fluorophosphate (DFP) demonstrated that DPPIV is a serine protease (Barth et al., 1974; Kenny et al., 1976). It is known that most of the serine proteases share the sequence Gly-X-Ser-X-Gly at the active site. Many serine endoproteases such as those of a trypsin family have the common sequence Gly-Asp-Ser-Gly-Gly (Dayhoff, 1978; Gershenfeld & Weissman, 1986; Brenner, 1988; Newport et al., 1988; Sakanari et al., 1989), although subtilisin-like endoproteases (Kraut, 1971; Mizuno et al., 1988; Hatsuzawa et al., 1990; Misumi et al., 1990c) and plant/yeast serine carboxypeptidases (Breddam et al., 1987; Liao & Remington, 1990) contain the atypical sequence Gly-X-Ser-X-Ala/Ser at the active site. The sequence Gly-X-Ser-X-Gly is also found in other serine-active hydrolases including esterases and lipases (Dayhoff, 1978; Brenner, 1988; Gibney et al., 1990; Winkler et al., 1990). The functional importance of the serine residue in the sequence has been confirmed by chemical modification and site-directed mutagenesis. However, no detailed information is available for possible involvement of other residues in the sequence Gly-X-Ser-X-Gly in their catalytic activities.

We have recently established the entire amino acid sequence of rat liver DPPIV deduced from the cDNA sequence (Ogata et al., 1989), demonstrating that it indeed contains *Gly*-Trp-*Ser*-Tyr-*Gly* at positions 629–633. In this study, we have used a [^{3}H]DFP labeling technique to identify Ser^{631} as the site of DFP modification. Furthermore, results obtained by site-specific mutagenesis revealed that all the residues Gly^{629}, Ser^{631}, and Gly^{633} are essential for enzyme activity of DPPIV.

Experimental Procedures

Materials. [^{3}H]Diisopropyl fluorophosphate (DFP) (3.0 Ci/mmol) and [^{35}S]methionine (>800 Ci/mmol) were obtained from Du Pont–New England Nuclear (Boston). Lysyl endopeptidase (*Achromobacter lyticus* protease I, EC 3.4.21.50) was obtained from Wako Junyaku (Osaka, Japan); peptide *N*-glycosidase F (*N*-glycanase) was from Genzyme Corp. (Boston); Gly-Pro-*p*-nitroaniline was from Protein Research Foundation (Osaka). Rabbit anti-(rat liver DPPIV) IgG was prepared as described previously (Ogata et al., 1989).

Purification of DPPIV. Plasma membranes were isolated from Wistar rat livers as described previously (Ray, 1970; Ikehara et al., 1977). The membranes were suspended in 20 mM Tris-HCl (pH 7.5) containing 0.5% (v/v) Triton X-100 and 1 mM $MgCl_2$ and treated with 1-butanol (finally 25%) at 4 °C for 1 h, followed by centrifugation at 15000*g* for 30 min (Misumi et al., 1990a). The resulting aqueous phase was used for purification of DPPIV by subjecting it to sequential chromatographies through concanavalin A–Sepharose, Sephacryl S-300, and wheat-germ lectin–Sepharose columns (Ogata et al., 1989). We finally obtained 5.6 mg of DPPIV with a specific activity of 59.4 units [μmol/(min·mg of protein)] from about 600 g of rat liver. The sample thus obtained was found to contain a single protein of 109 kDa when it was analyzed by SDS–PAGE.

Affinity Labeling of DPPIV. Purified DPPIV (2 mg) was incubated at 25 °C for 2 h with 250 μCi of [^{3}H]DFP in 2 mL of 50 mM Tris-HCl (pH 8.0) containing 0.1% Triton X-100 and 0.02% (w/v) NaN_3. After the addition of 2 μmol of unlabeled DFP into the reaction mixture, the incubation continued at 4 °C for an additional 24 h to achieve complete inhibition and aging. The aging process which results in the loss of one of the isopropyl groups was necessary to ensure that a single species conjugated to the serine (MacPhee-Quigley et al., 1985). After the removal of the unreacted DFP by dialysis, the sample was lyophilized and subjected to ethanol precipitation to remove Triton X-100 as completely as possible (Ogata et al., 1987, 1988).

When being analyzed by SDS–PAGE/fluorography, samples including cells and plasma membranes were incubated at 25 °C for 1 h with [^{3}H]DFP (3.0 Ci/mmol) in the absence of unlabeled DFP, for obtaining DPPIV with higher specific radioactivities.

Isolation and Sequencing of the [^{3}H]DFP-Labeled Peptide. The [^{3}H]DFP-labeled DPPIV (1 mg) was cleaved by incubation with lysyl endopeptidase (enzyme:substrate ratio by weight, 1:100) at 37 °C for 6 h in 0.3 mL of 50 mM Tris-HCl (pH 9.0) containing 4 M urea (Tsunasawa et al., 1987; Ogata et al., 1990). The resultant peptide fragments were freeze-dried and then subjected to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 × 25 cm) with a linear gradient from 10 to 50% (v/v) acetonitrile in 0.1% trifluoroacetic acid. The elution profile was monitored by the absorbance at 215 nm, and the ^{3}H radioactivity distribution was determined. Fractions of a major radioactivity peak were collected and freeze-dried. About 300 pmol of the ^{3}H-labeled peptide thus obtained was subjected to the automated Edman degradation on an Applied Biosystems Inc. Model 477A gas-phase sequencer (Ogata et al., 1989). Each cycle yielded 150 μL of the degradation product in a conversion flask, of which 50 μL was used for the identification of the product by an on-line Model 120A phenylthiohydantoin derivative analyzer, and the remaining sample (100 μL) was used for determination of the radioactivity.

Construction of Expression Plasmids and Site-Directed Mutagenesis. The cDNA insert of wild-type rat DPPIV was prepared (Ogata et al., 1989) and inserted into the *Eco*RI site of a pSG5 expression vector (Green et al., 1988) (designated as pSGDP). A uracil-containing antisense strand of the plasmid was prepared from *Escherichia coli* BW313 and used as a template for site-directed mutagenesis (Kunkel, 1985). An 18-mer synthetic oligonucleotide (5′-pAATTTGGGCCTGGTCATA-3′) was used as a primer for in vitro synthesis of the second strand. The mutant plasmid thus obtained was designated as pSGDP/GA-629, which encodes a DPPIV variant with substitution of Gly^{629}(GGC) → Ala(G*C*C).

The following eight other mutant plasmids were also prepared by using 18-mer oligonucleotides (single substitutions) or 28-mer oligonucleotides (double substitutions) as primers: pSGDP/GR-629, Gly^{629}(GGC) → Arg(*C*GC); pSGDP/WE-630, Trp^{630}(TGG) → Glu(*GA*G); pSGDP/SA-631, Ser^{631}(TCA) → Ala(*G*CA); pSGDP/YF-632, Tyr^{632}(TAT) → Phe(T*T*T); pSGDP/YL-632, Tyr^{632}(TAT) → Leu(T*TG*); pSGDP/YG-632, Tyr^{632}(TAT) → Gly(*GG*T); pSGDP/GA-633, Gly^{633}(GGA) → Ala(G*C*A); pSGDP/GS, Gly^{633}(GGA) → Ser(*TC*A). The nucleotide sequences of these mutant

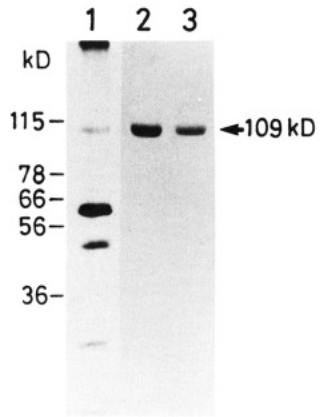

FIGURE 1: SDS-PAGE/fluorography of [^{3}H]DFP-labeled DPPIV. Samples which had been labeled under the indicated conditions were analyzed by SDS–PAGE (9.0% gels) followed by fluorography. Lane 1, rat liver plasma membranes (30 μg) labeled with [^{3}H]DFP (25 μCi) at 37 °C for 1 h; lane 2, purified DPPIV (1.4 μg) labeled with [^{3}H]DFP (15 μCi) at 37 °C for 1 h; lane 3, [^{35}S]methionine-labeled DPPIV prepared by immunoprecipitation from hepatocyte lysates. Marker proteins used are the α (115 kDa) and β (66 kDa) chains of complement C3, transferrin (78 kDa), α_1-protease inhibitor (56 kDa), and haptoglobin β chain (36 kDa).

clones were confirmed by the dideoxynucleotide chain termination method (Sanger et al., 1977).

Transfection and Analysis of Expressed Proteins. Each plasmid (10 μg) was transfected into 5×10^6 COS-1 cells using an electroporation apparatus (Gene Pulser, Bio-Rad) as described previously (Oda et al., 1989). The transfected cells were cultured in Dulbecco's-modified Eagle's medium containing 10% (v/v) fetal calf serum in 10-cm dishes for 2 days before the following experiments. The cells were incubated at 37 °C for 3 h either with [^{3}H]DFP (60 μCi/dish) in 5 mL of the Eagle's minimum essential medium or with [^{35}S]-methionine (60 μCi/dish) in 5 mL of the Eagle's medium lacking methionine. After removal of the medium, the cells were washed with Dulbecco's phosphate-buffered saline (PBS) and lysed in 0.5 mL of PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS, followed by centrifugation at 15000g for 20 min. ^{3}H- or ^{35}S-labeled DPPIV in each supernatant was immunoprecipitated with anti-DPPIV IgG and analyzed by SDS–PAGE(9.0% gels)/fluorography (Takami et al., 1988; Ogata et al., 1989).

Other Methods. ^{35}S-Labeled DPPIV was digested at 37 °C for 18 h with *N*-glycanase (10 units/mL) in 0.1 M phosphate buffer (pH 8.6) (Plummer et al., 1984). DPPIV activity was assayed with Gly-Pro-*p*-nitroaniline as a substrate (Nagatsu et al., 1976). Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The primary culture of rat hepatocytes and metabolic labeling with [^{35}S]methionine were carried out as described previously (Misumi et al., 1986).

RESULTS

Affinity Labeling of DPPIV with [^{3}H]DFP. It was shown that the incubation of DPPIV with the serine-modifying reagent DFP results in the complete loss of enzyme activity (Barth et al., 1974; Kenny et al., 1976). However, no information is available for the location of the active site serine in the primary structure. For identification of the active site serine, we took advantage of affinity labeling of DPPIV with [^{3}H]DFP. Purified DPPIV was labeled with [^{3}H]DFP and subjected to SDS–PAGE/fluorography, demonstrating a single component with M_r 109 000 (Figure 1, lane 2). The protein migrated to the same position as that obtained by immunoprecipitation from hepatocytes metabolically labeled with [^{35}S]methionine (Figure 1, lane 3), indicating the intactness of the purified protein. In addition, DPPIV in the rat liver plasma membrane was found to be a relatively minor component of four proteins labeled with [^{3}H]DFP (Figure 1, lane 1), in contrast to the finding that DPPIV is the only protein labeled with [^{32}P]DFP in the kidney brush-border membrane (Kenny et al., 1976, 1987).

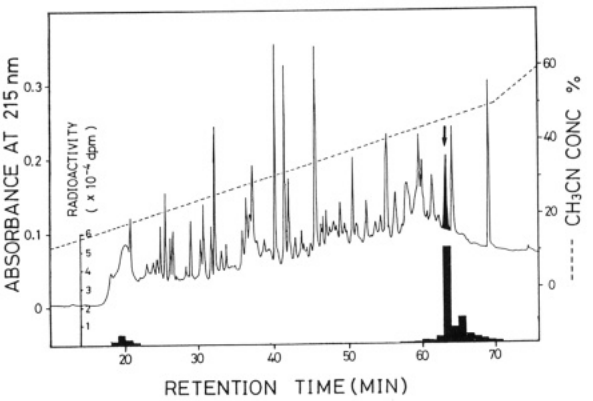

FIGURE 2: HPLC of DPPIV-derived peptides for isolation of a [^{3}H]DFP-labeled peptide. [^{3}H]DFP-labeled DPPIV was cleaved with lysyl endopeptidase, and the digest was subjected to reverse-phase HPLC on a TSKgel ODS-120T column with a linear gradient from 10 to 50% acetonitrile (dashed line) in 0.1% trifluoroacetic acid. The elution profile was monitored by absorbance at 215 nm (solid line), and the distribution of ^{3}H radioactivity was determined (closed bar). Fractions of a major radioactivity peak (indicated by an arrow) were pooled and used for amino acid sequencing.

Isolation and Sequencing of [^{3}H]DFP-Labeled Peptide. The [^{3}H]DFP-labeled DPPIV was cleaved with lysyl endopeptidase, and the digest was subjected to reverse-phase HPLC, followed by determination of the ^{3}H radioactivity (Figure 2). A fraction with the highest radioactivity (indicated by an arrow) was found to contain a single radioactive component with M_r 2700, when analyzed by SDS–PAGE/fluorography (data not shown). The sample was subjected to an analysis of the amino acid sequence. The sequence started with Arg and was definitely identified up to Phe at cycle 25, although a residue at cycle 8 remained unidentified (Table I). The highest radioactivity was found in the unidentified residue at cycle 8, indicating its modification with [^{3}H]DFP. A comparison with the primary structure of DPPIV predicted by the cDNA sequence (Ogata et al., 1989) revealed that the sequence determined here is assigned to that at positions 624–648 in the predicted sequence and that the position X is occupied by Ser^{631}. Thus, it is concluded that Ser^{631} is the residue that was modified with [^{3}H]DFP. The sequence Gly-Trp-Ser-Tyr-Gly surrounding the [^{3}H]DFP-modified Ser^{631} corresponds to the active site sequence Gly-X-Ser-X-Gly commonly observed in serine hydrolases (Dayhoff, 1978; Brenner, 1988).

Site-Directed Mutagenesis and Expression of Mutated cDNAs. When the cDNA for the wild-type DPPIV was transfected into COS-1 cells, the protein expressed was found to have an apparent molecular mass of about 105 kDa (Figure 3; panel B, lane 2, and panel D, lane 5), slightly smaller than that (109 kDa) of the enzyme in rat hepatocytes (Figure 3D, lane 6). DPPIV has eight potential N-glycosylation sites (Ogata et al., 1989), of which at least six sites are actually glycosylated (Yamashita et al., 1988), but it contains no O-linked oligosaccharide chains (Yamashita et al., 1988). After treatment with *N*-glycanase, the 105-kDa and 109-kDa forms migrated to the same 84-kDa position (Figure 4, lanes 1 and 5). Thus, it is likely that the difference in their mo-

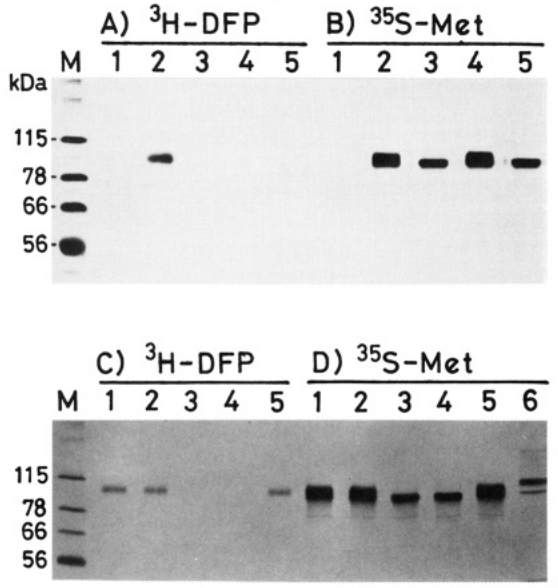

FIGURE 3: Analysis of DPPIV expressed in the transfected cells. COS-1 cells were transfected with the indicated plasmids and cultured at 37 °C for 2 days. The cells were incubated at 37 °C for 3 h in the presence of [^{3}H]DFP (60 μCi/dish) (panels A and C) or [^{35}S]-methionine (60 μCi/dish) (panels B and D). The cells were washed and lysed, followed by immunoprecipitation with anti-DPPIV IgG. The immunoprecipitates were analyzed by SDS–PAGE (9.0% gels)/fluorography. Panels A and B: lane 1, mock; lane 2, pSGDP/wild; lane 3, pSGDP/GA-629; lane 4, pSGDP/SA-631; lane 5, pSGDP/GA-633. Panels C and D: lane 1, pSGDP/WE-630; lane 2, pSGDP/YF-632; lane 3, pSGDP/YL-632; lane 4, pSGDP/YG-632; lane 5, pSGDP/wild; lane 6, ^{35}S-labeled DPPIV obtained from a primary culture of rat hepatocytes. Marker proteins (M) include the α (115 kDa) β (66 kDa) chains of complement C3, transferrin (78 kDa), and α_1-protease inhibitor (56 kDa) which were metabolically labeled with [^{35}S]methionine.

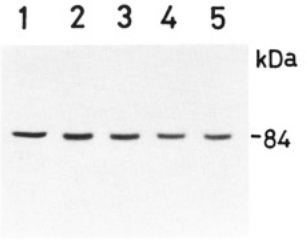

FIGURE 4: *N*-Glycanase treatment of DPPIV expressed in COS-1 cells and hepatocytes. Samples of ^{35}S-labeled DPPIV were prepared as shown in Figure 3 and were treated with *N*-glycanase (10 units/mL, at 37 °C for 18 h). Aliquots of the treated samples were adjusted to contain the same radioactivity (3000 dpm/lane) and were subjected to SDS–PAGE/fluorography. Lane 1, pSGDP/wild; lane 2, pSGDP/GA-629; lane 3, pSGDP/SA-631; lane 4, pSGDP/GA-633; lane 5, rat hepatocyte DPPIV.

lecular masses is due to a difference in the extent of N-glycosylation between the COS-1 cells and the hepatocytes (Misumi et al., 1990b). The wild-type DPPIV expressed on the cell surface was also identified by modification with [^{3}H]DFP (Figure 3; panel A, lane 2, and panel C, lane 5).

To confirm further essential functional residues at the active site of DPPIV, we modified the cDNA by site-directed mutagenesis. We designed the initial study to cause single mutations at the consensus sequence Gly-X-Ser-X-Gly (Gly^{629} → Ala, Ser^{631} → Ala, and Gly^{633} → Ala). Transfection and expression analyses revealed that any single substitution of these residues resulted in production of the mutant forms which cannot be modified with [^{3}H]DFP (Figure 3A,B). No increase in enzyme activity was observed in all the transfected cells, in contrast to a remarkable increase of the activity in the cells

Table I: Amino Acid Sequence and Radioactivity Distribution of the [^{3}H]DFP-Labeled Peptide[a]

cycle no.	PTH-amino acid	yield (pmol)	radioactivity (dpm)	predicted[b]
1	Arg	96	450	Arg
2	Val	223	580	Val
3	Ala	119	190	Ala
4	Ile	105	480	Ile
5	Trp	47	390	Trp
6	Gly	103	280	Gly
7	Trp	20	640	Trp
8	X[c]		15740	Ser
9	Tyr	47	3380	Tyr
10	Gly	96	1140	Gly
11	Gly	119	530	Gly
12	Tyr	41	380	Tyr
13	Val	40	290	Val
14	Thr	54	280	Thr
15	Ser	28	1040	Ser
16	Met	27	320	Met
17	Val	31	210	Val
18	Leu	32	190	Leu
19	Gly	56	200	Gly
20	Ser	9	180	Ser
21	Gly	43	210	Gly
22	Ser	4	140	Ser
23	Gly	13	310	Gly
24	Val	13	160	Val
25	Phe	12	220	Phe

[a] The [^{3}H]DFP-labeled peptide was prepared as shown in Figure 2 and was subjected to amino acid sequencing, followed by determination of the radioactivity as described under Experimental Procedures. PTH, phenylthiohydantoin; dpm, disintegrations per minute. [b] Amino acid residues at positions 624–648 predicted by the cDNA sequence (Ogata et al., 1989). [c] No amino acid was identified.

Table II: Enzyme Activities of DPPIV in Transfected COS-1 Cells[a]

transfected plasmid	mutation	enzyme activity (μmol/(min·mg of protein))	[^{3}H]DFP binding[b]
mock		0.002	–
pSGDP/wild		0.520	+
pSGDP/GA-629	Gly^{629} → Ala	0.001	–
pSGDP/GR-629	Gly^{629} → Arg	0.002	–
pSGDP/WE-630	Trp^{630} → Glu	0.488	+
pSGDP/SA-631	Ser^{631} → Ala	0.001	–
pSGDP/YF-632	Tyr^{632} → Phe	0.452	+
pSGDP/YL-632	Tyr^{632} → Leu	0.029	–
pSGDP/YG-632	Tyr^{632} → Gly	0.002	–
pSGDP/GA-633	Gly^{633} → Ala	0.001	–
pSGDP/GS-633	Gly^{633} → Ser	0.001	–

[a] COS-1 cells were transfected with the indicated plasmids and cultured at 37 °C for 2 days. The cells were washed and lysed in PBS containing 1% Triton X-100, followed by determination of DPPIV activity with Gly-Pro-*p*-nitroaniline as a substrate. Values are the means of those obtained from two separate experiments. [b] Data taken from the labeling experiments (see Figure 3).

transfected with the wild-type cDNA (Table II). It was also confirmed that other substitutions (Gly^{629} → Arg and Gly^{633} → Ser) gave the same results as above (Table II).

Further experiments were carried out with mutants having substitutions of X residues in the consensus sequence (Figure 3C,D). Substitution of Trp^{630} → Glu had no significant effect on the modification of the expressed protein with [^{3}H]DFP (Figure 3C, lane 1) and on the expression of enzyme activity (Table II). Substitution of Tyr^{632}, however, caused contrasting effects on the enzyme activity depending on the residues used as the substituents. A mutant of Tyr^{632} → Phe retained essentially the same reactivity with [^{3}H]DFP (Figure 3C, lane

2) and enzyme activity as those of the wild-type, whereas substitution of Tyr^{632} by Leu or Gly resulted in the complete loss in DFP reactivity of the expressed mutants (Figure 3C, lanes 3 and 4), which had little or no enzyme activity (Table II).

Electrophoretic profiles (Figure 3B,D) show that the mutants expressed in COS-1 cells do not have completely the same molecular mass. When being treated with *N*-glycanase, all the mutant forms migrated to the same 84-kDa position as that of the wild-type from COS-1 cells and hepatocytes, as some examples show in Figure 4. This may also support the involvement of oligosaccharides in the difference in molecular mass and broadness of the expressed forms. It is, however, unclear at present why these forms are differently glycosylated despite being produced in the same COS-1 cells.

Discussion

In the present study, we determined the amino acid sequence of the [^{3}H]DFP-labeled peptide prepared from DPPIV and compared it with the entire sequence predicted by the cDNA sequence (Ogata et al., 1989), demonstrating that Ser^{631} is modified with DFP, a potent inhibitor of the enzyme. Site-directed mutagenesis changing Ser^{631} to Ala in DPPIV abolished its catalytic activity and interaction with [^{3}H]DFP, establishing Ser^{631} as the active site serine in DPPIV. This serine residue was found in the sequence Gly-Trp-Ser-Tyr-Gly, which corresponds to the active site motif Gly-X-Ser-X-Gly (G_1-X_1-S-X_2-G_2) proposed for serine proteases.

In addition to the active serine, functional roles of other residues in the G_1-X_1-S-X_2-G_2 sequence were examined by site-directed mutagenesis of the cDNA and transfection/expression experiments. Gly at position G_1 is strictly conserved in all serine hydrolases, including proteases, esterases, and lipases, for which the active-site sequence has been identified. Position G_2, however, is not always occupied by Gly, which is replaced by Ala or Ser in several serine proteases such as subtilisins (Kraut, 1971), Kex2 (Mizuno et al., 1988), furin (Hatsuzawa et al., 1990; Misumi et al., 1990) and plant/yeast serine carboxypeptidases (Sørensen et al., 1987; Breddam et al., 1987). The observations suggest the possibility that the stringency in the requirement of Gly is different between G_1 and G_2; any of the residues (Gly, Ala, or Ser) having simple side-chain structure are replaceable at position G_2 without functional change. At least in the case of DPPIV, however, this possibility has been ruled out. Substitution of Gly^{633} (G_2 position) with Ala or Ser resulted in the complete loss of enzyme activity, as observed for substitution of Gly^{629} (G_1 position). The results indicate that Gly is absolutely required for both positions G_1 and G_2 at the active site of DPPIV.

Positions X_1 and X_2 contain various residues in serine hydrolases (Brenner, 1988), suggesting that residues at these positions are not essential for catalytic activity. In fact, at position X_1 of DPPIV, substitution of $Trp^{630} \rightarrow$ Glu, a drastic change from a neutral and bulky residue to a negatively charged residue, caused no effect on enzyme activity of the expressed molecule. In contrast, a different response was observed by substitution of Tyr^{632} at position X_2. The residue Phe, structurally similar to Tyr, could be substituted without significant effect on the enzyme activity. However, substitution by Leu or Gly failed to retain the activity, although these residues are found at the corresponding position in many other serine hydrolases (Gershenfeld & Weissman, 1986; Brenner, 1988; Newport et al., 1988; Sakanari et al., 1989). Thus, it is likely that the residue Tyr^{632} is also important in the active site of DPPIV. The same residue is found at position X_2 of the consensus sequence in plant/yeast carboxypeptidases (Sørensen et al., 1986, 1987; Breddam et al., 1987).

The active center of serine proteases is composed not only of Ser but also of His and Asp, that is, a catalytic triad (Kraut, 1971; Gershenfeld & Weissman, 1986; Sakanari et al., 1989). The same catalytic triad (His, Asp, and Ser) has been proposed for the active center of other serine hydrolases including lipases (Brady et al., 1990; Gibney et al., 1990; Winkler et al., 1990), although not so extensively studied as for the serine proteases. The His and Asp residues of the trypsin family are conserved in the sequences A-A-*H*-C and *D*-I-X-L, respectively, while those of the subtilisin family are in the sequences *H*-G-T and *D*-D/S-G, respectively. Although no direct evidence has been obtained for involvement of His and/or Asp residues in DPPIV activity, the same sequence *H*-G-T is found at positions 705–707 in the predicted sequence of DPPIV. A location of the putative Asp residue in the sequence potentially fulfilling the triad remains to be determined.

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Registry No. DPPIV, 54249-88-6; Ser, 56-45-1; Gly, 56-40-6; Tyr, 60-18-4; Leu, 61-90-5.

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