An Active-Site Mutation ($Gly^{633} \rightarrow Arg$) of Dipeptidyl Peptidase IV Causes Its Retention and Rapid Degradation in the Endoplasmic Reticulum†

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ABSTRACT: Dipeptidyl peptidase IV (DPPIV), a serine protease expressed on the cell surface, is deficient in a Fischer rat substrain. Northern blot analysis showed no difference in the size and amount of DPPIV mRNA between normal (344/NC) and deficient (344/CRJ) rats. Cloning and sequencing of DPPIV cDNAs revealed a G to A transition at nucleotide 1897 in the cDNA sequence of 344/CRJ, which leads to substitution of $Gly^{633} \rightarrow Arg$ in the active-site sequence Gly^{629}-Trp-Ser-Tyr-Gly^{633} determined for the wild-type DPPIV [Ogata, S., Misumi, Y., Takami, N., Oda, K., & Ikehara, Y. (1992) *Biochemistry 31,* 2582–2587]. Pulse–chase experiments with hepatocytes showed that the wild-type DPPIV was initially synthesized as a 103-kDa form with high-mannose-type oligosaccharides, which was processed to a mature form of 109 kDa with the complex type during intracellular transport. In contrast, the mutant DPPIV, although being synthesized as the 103-kDa form, was rapidly degraded without being processed to the mature form. Site-directed mutagenesis of the wild-type and mutant cDNAs and their transfection/expression in COS-1 cells confirmed that the single substitution of $Gly^{633} \rightarrow Arg$ is sufficient to cause the rapid intracellular degradation of DPPIV. Immunoelectron-microscopic observations showed that the mutant DPPIV was detectable only in the endoplasmic reticulum (ER), in contrast to the distribution of the wild-type DPPIV in the Golgi complex and on the cell surface. Taken together, these results suggest that the substitution of $Gly^{633} \rightarrow Arg$ at the active site causes a conformational change of DPPIV coupled with its rapid degradation in the ER, accounting for the enzyme defect in the substrain 344/CRJ.

Dipeptidyl peptidase IV (DPPIV)[1] shows marked preference for the cleavage of prolyl bonds, and is expressed as an ectoenzyme on the cell surface, in contrast to other DPPs localized in the lysosome (DPPI and DPPII) and in the cytoplasm (DPPIII) (Hapsu-Havu & Glenner, 1966; McDonald & Schwabe, 1977; Macnair & Kenny, 1979). In polarized epithelial cells in the liver, small intestine, and kidney proximal tubules, DPPIV is localized in the apical domain of the cell surface (Fukasawa et al., 1981; Bartles et al., 1985; Hartel et al., 1988). This is accomplished by a sorting event of newly synthesized DPPIV occurring during its transport from the Golgi complex to the cell surface (Low et al., 1991; Casanova et al., 1991) or by its transcytosis after reaching the basolateral domain (Matter et al., 1990), depending upon cell types examined. Such a subcellular localization and high levels of the enzyme activity suggest that DPPIV is involved in the metabolism and uptake of proline-containing peptides in these tissues (Kreil et al., 1980; Miyamoto et al., 1987; Tiruppathi et al., 1990a).

On the basis of the cDNA sequence for DPPIV, we have demonstrated that its primary structure consists of 767 amino acid residues and has 8 potential sites for N-linked glycosylation (Ogata et al., 1989), while Hong and Doyle (1987) proposed a slightly different structure with a COOH-terminal extension (792 residues). It is now established that DPPIV is anchored to the membrane by its NH_2-terminal uncleavable signal sequence (Ogata et al., 1989; Hong & Doyle, 1990) and has the same feature in membrane topology (type II) as several other hydrolases in brush-border membranes such as γ-glutamyl transpeptidase (Laperche et al., 1986), sucrase-isomaltase (Hunziker et al., 1986), and neutral endoprotease (Devault et al., 1987). In addition, the active-site serine of DPPIV has been identified at position 631 by affinity labeling with [^{3}H]diisopropyl fluorophosphate ([^{3}H]DFP) and by site-directed mutagenesis (Ogata et al., 1992). The sequence Gly-Trp-Ser-Tyr-Gly (positions 629–633) surrounding the active serine-631 corresponds to the consensus sequence Gly-X-Ser-X-Gly proposed for the active site of serine proteases (Brenner, 1988).

Watanabe et al. (1987) reported that DPPIV activity was markedly reduced in membranes prepared from tissues of Fischer-344 rats as compared with that of Wistar rats. The lack in enzyme activity and immunological reactivity of DPPIV was confirmed by other groups (Gassrau et al., 1990; Thompson et al., 1991; Tiruppathi et al., 1990a). In the present study, we have examined the molecular basis for the enzyme deficiency in the Fischer rat substrain 344/CRJ, demonstrating that a single substitution of $Gly^{633} \rightarrow Arg$ at the active-site sequence causes retention and rapid regradation of the mutant DPPIV in the endoplasmic reticulum (ER).

EXPERIMENTAL PROCEDURES

Materials. [^{35}S]Methionine (900 Ci/mmol) and [^{3}H]DFP (3.0 Ci/mmol) were obtained from Du Pont–New England Nuclear. The Sequenase DNA-sequencing kit was from United States Biochemical Corp. Various DNA-modifying enzymes and restriction endonucleases were obtained from Nippon Gene (Toyama, Japan) and Takara Shuzo (Kyoto, Japan); endoglycosidase H was from Seikagaku Kogyo

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[1] Abbreviations: DPPIV, dipeptidyl peptidase IV; DFP, diisopropyl fluorophosphate; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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(Tokyo); Gly-Pro-*p*-nitroaniline was from Protein Research Foundation (Osaka). Brefeldin A was kindly provided by Dr. E. Taniguchi (Kyushu University). Anti-DPPIV antiserum was prepared as described previously (Ogata et al., 1989).

Rat Substrains. Fischer rat substrains, 344/CRJ and 344/NC, were obtained from Charles River Japan, Inc. (Atsugi, Japan), and Nihon Clea, Inc. (Osaka), respectively.

Preparation of Plasma Membranes and [^{3}H]DFP-Labeled DPPIV. Plasma membranes were isolated from liver and kidney of the two substrains as described previously (Ikehara et al., 1977). Each sample of the plasma membranes (500 μg) was incubated at 37 °C for 1 h with 250 μCi of [^{3}H]DFP in 1 mL of 50 mM Tris-HCl (pH 8.0), followed by centrifugation at 35000*g* for 30 min. The resulting membrane pellets were suspended in 1 mL of phosphate-buffered saline (PBS) containing 1% Triton X-100, and the suspension was mixed well for 30 min followed by centrifugation at 12000*g* for 10 min. The supernatant obtained was used for immunoprecipitation of [^{3}H]DFP-labeled DPPIV (Ogata et al., 1992).

Hepatocyte Culture and Pulse–Chase Experiments. Hepatocytes were isolated from livers of the wild (344/NC) and mutant (344/CRJ) rats and cultured as described previously (Oda et al., 1983). After a 24-h culture, the cells (2×10^6 cells/60-mm dish) were incubated at 37 °C for 30 min in Eagle's minimum essential medium lacking methionine. The cells were pulse-labeled for 20 min with [^{35}S]methionine (100 μCi/dish). At the indicated times of chase, cell lysates were prepared and subjected to immunoprecipitation of DPPIV. When indicated, cells were incubated in the presence of brefeldin A (5 μg/mL) during pulse–chase periods.

SDS–PAGE and Fluorography. SDS–PAGE (9% gels) was carried out according to Laemmli (1970), followed by fluorography. Apparent molecular weights were determined by coelectrohoresis of marker proteins metabolically labeled with [^{35}S]methionine (Misumi et al., 1986): α (115K) and β (65K) chains of rat complement C3, transferrin (78K), and α_1-protease inhibitor (56K). In some cases, fluorograms were scanned at 540 nm using a Shimadzu CS-900 densitometer. The area under the resulting peaks was integrated and used as a quantitative measure for the radioactivity in the bands (Takami et al., 1990).

Cloning and Sequencing of DPPIV cDNAs. Poly(A)$^+$ RNA fractions were prepared from kidneys of the 344/NC and 344/CRJ rats and used for construction of the kidney cDNA library for each substrain as described previously (Misumi et al., 1990). The resultant cDNA libraries were screened by the hybridization method (Maniatis et al., 1982) using as a probe *Eco*Ri–*Bgl*II fragment (368 bp) of Wistar rat liver DPPIV cDNA (λcDp37) (Ogata et al., 1989). Screening of 3×10^5 clones from the 344/NC library, without prior amplification, yielded more than 80 positive clones, of which 12 clones were processed to the second screening. In the case of the 344/CRJ library, 5×10^5 clones were screened, yielding more than 120 positive clones, of which 12 clones were subjected to the second screening. Two cDNA clones with the longest insert (3.5 kb) obtained from each library were subcloned into pUC118 plasmid vector: pcDPF for Fischer-344/NC (wild) and pcDPFM for 344/CRJ (mutant). pcDPF and pcDPFM were treated with exonuclease III and mung bean nuclease to obtain unidirectional-deletion subclones as described (Hoheisel & Pohl, 1986). After single-stranded DNAs were isolated with the aid of helper phages (M13K07), both strands of all regions were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977) using the Sequenase DNA-sequencing kit.

Northern Blot Analysis. Poly(A)$^+$ RNA (6 μg of each) prepared from liver and kidney was denatured with 50% formamide/6.5% formaldehyde and electrophoresed on 1.1% agarose gels containing 6% formaldehyde. The separated RNA was transferred to a Durapore membrane (Millipore) and hybridized with ^{32}P-labeled *Eco*RI–*Bgl*II fragment of λcDP37 (Ogata et al., 1989) which had been prepared with a multiprime DNA labeling system (Feinberg & Vogelstein, 1983).

Construction of Expression Plasmids and Site-Directed Mutagenesis. cDNA inserts of pcDPF (wild) and pcDPFM (mutant) were prepared by digestion of each plasmid with *Eco*RI, electrophoretic purification, and then insertion into the *Eco*RI site of the pSG5 expression vector (Green et al., 1988). Each insert orientation was confirmed by restriction endonuclease mapping. The expression plasmids thus prepared were designated as pSD (wild) and pSDM (mutant). These plasmids were modified by site-directed mutagenesis to encode DPPIV molecules in which Gly (wild) and Arg (mutant) at position 633 were substituted by each other. Antisense strands of each plasmid were used as a template for site-directed mutagenesis (Kunkel, 1988). 18-mer synthetic oligonucleotides, 5′-pGTCATATAGAGGGTACGT-3′ and 5′-pGTCATATGGAGGGTACGT-3′, were used as a primer for in vitro synthesis of the second strand of pSD and pSDM, respectively. The plasmids thus obtained were designated as pSD/GR, which encodes DPPIV with substitution of Gly^{633} (GGA) → Arg (AGA), and as pSDM/RG, encoding the protein with substitution of Arg^{633} (AGA) → Gly (GGA).

Transfection and Analysis of Expressed Proteins. Each plasmid (20 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 Earle's medium containing 10% fetal calf serum in 10-cm dishes for 2 days before the following experiments. The cells were pulse-labeled for 20 min with [^{35}S]methionine (50 μCi/dish) in the Eagle's minimum essential medium lacking unlabeled methionine and then chased. At the indicated times of chase, cell lysates were prepared and subjected to immunoprecipitation of ^{35}S-labeled DPPIV, followed by SDS–PAGE and fluorography as described above.

Immunoelectron Microscopy. Transfected cells were fixed for 2 h at room temperature with the periodate/lysine/paraformaldehyde fixative (Fujiwara et al., 1988) and washed with PBS. After being permeabilized with 0.1% saponin in PBS for 20 min, cells were incubated at room temperature for 1 h each first with rabbit anti-DPPIV IgG and then with peroxidase-conjugated goat Fab of anti-rabbit IgG (Fujiwara et al., 1988). The peroxidase reaction was carried out at room temperature for 15 min in a substrate medium containing diaminobenzidine (0.5 mg/mL) and 0.01% H_2O_2. The cells were processed for electron microscopy as described previously (Fujiwara et al., 1988).

Other Methods. DPPIV activity was determined with Gly-Pro-*p*-nitroanilide as a substrate (Nagatsu et al., 1976). Digestion of ^{35}S-labeled DPPIV with endoglycosidase H (0.2 unit/mL) was carried out at 37 °C for 16 h in 50 mM acetate buffer (pH 5.5).

RESULTS

Defect of DPPIV in a Fischer Rat Substrain. Plasma membranes of liver and kidney were prepared from the

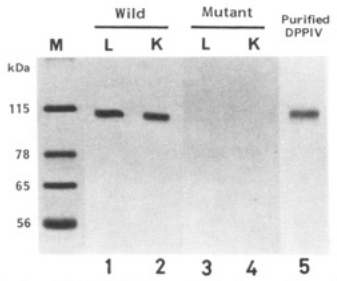

FIGURE 1: Defect of DPPIV in a Fischer rat substrain. Plasma membranes of liver (L) and kidney (K) from the substrain 344/NC (wild) and 344/CRJ (mutant) were labeled with [^{3}H]DFP, dissolved in 1% Triton X-100, and subjected to immunoprecipitation with anti-DPPIV IgG. The immunoprecipitates were analyzed by SDS–PAGE and fluorography. Lane M, molecular mass markers including the α (115 kDa) and β (kDa) chains of rat complement C3, transferrin (78 kDa), and α_1-protease inhibitor (56 kDa). Lane 5, DPPIV purified from the wild-type rat liver and stained with Coomassie brilliant blue.

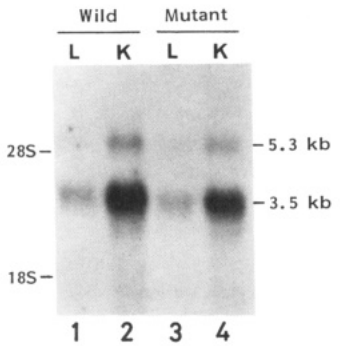

FIGURE 2: RNA blot analysis of DPPIV mRNA. Poly(A)$^+$ RNAs (6 μg of each) prepared from liver (L) and kidney (K) of the wild and mutant rats were denatured and separated by electrophoresis on a 1.1% agarose gel. The RNA was blotted onto a Durapore membrane and hybridized with the ^{32}P-labeled *Eco*RI–*Bgl*II fragment prepared from the Wistar rat DPPIV cDNA λcDP37. RNA size markers are 28S and 18S rRNAs of rat liver.

substrains 344/NC and 344/CRJ, and labeled with [^{3}H]DFP, a potent inhibitor of DPPIV covalently linking with the active-site serine. [^{3}H]DFP-labeled DPPIV obtained by immunoprecipitation was analyzed by SDS–PAGE (Figure 1). While a single band of 109 kDa was easily identified in each sample of the liver (lane 1) and kidney (lane 2) membranes from 344/NC, no radioactive band was detectable in the membranes from 344/CRJ (Figure 1, lanes 3 and 4). It was also confirmed that no immunoprecipitin line was formed between anti-DPPIV IgG and Triton X-100 extracts of the membranes from 344/CRJ, when analyzed by an immunodiffusion method (data not shown). These results indicate that DPPIV is defective in the tissues of Fischer-344/CRJ.

Identification of the Mutation Site in DPPIV. A possible change in the mRNA level of DPPIV was examined by Northern blot analysis. As shown in Figure 2, a major RNA of 3.5 kb was identified in the two tissues, although the mRNA level of kidney was much higher than that of liver. However, no significant difference in size and amount of the mRNA was observed between the two substrains, when compared in each tissue. Although an additional minor band of 5.3 kb was seen in each kidney preparation (Figure 2, lanes 2 and 4), this band was also detectable in the liver, when the membrane was exposed for much longer times, or when larger amounts of the liver RNA were used for analysis. In a previous study (Ogata et al., 1989), we isolated two cDNA clones (λcDP37 and λcD5) for Wistar rat liver DPPIV, of which λcD5 had a much longer 3′-noncoding region (about 1.5 kb). The major mRNA identified here corresponds to λcDP37, while the minor one may correspond to λcD5.

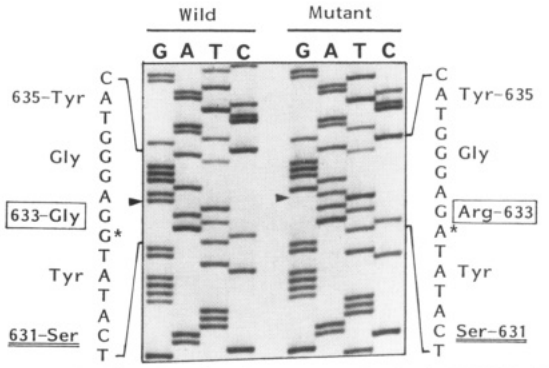

FIGURE 3: Identification of a missense mutation in the DPPIV cDNA (344/CRJ). Shown in the center are autoradiograms of the sequencing gels. Left, the wild-type DPPIV sequence around Gly^{633} (344/NC). Right, the sequence of the same region of the mutant DPPIV (344/CRJ). The nucleotide sequences are indicated along the corresponding amino acids and residue numbers. Nucleotide G* (also indicated by an arrowhead on the gel) in the codon for Gly^{633} (GGA) is substituted by nucleotide A*, resulting in formation of a codon for Arg^{633} (AGA) in the mutant DPPIV. Ser^{631} (double-underlined) represents the active-site serine.

We then isolated and sequenced the cDNA clones for DPPIV of the two substrains. Each cDNA insert contained an open reading frame encoding a 767 amino acid polypeptide, the same residue number determined for DPPIV of the Wistar rat liver (Ogata et al., 1989). The amino acid sequence deduced from the wild-type cDNA (pcDPF from 344/NC) was completely identical with that predicted by the Wistar rat cDNA (pcDP37), although two silent mutations were found at nucleotides 633 (A → G) and 750 (C → G). In contrast, the cDNA from 344/CRJ (pcDPFM) had an additional mutation at nucleotide 1897 (G → A), resulting in substitution of Gly with Arg at position 633 in the amino acid sequence, as shown in Figure 3. The Gly residue at position 633 is a residue found in the active-site sequence Gly^{629}-Trp-Ser-Tyr-Gly^{633} identified for DPPIV (Ogata et al., 1992).

Rapid Degradation of the Mutant DPPIV in Cultured Hepatocytes. The mutation Gly^{633} → Arg at the active-site sequence may result in production of an inactive form of DPPIV, accounting for no enzyme activity detectable in the affected rat tissues, but not providing a direct explanation for the absence of the immunoreactive antigen in the isolated plasma membrane. To clarify this point, we carried out pulse–chase experiments of hepatocytes with [^{35}S]methionine. The wild-type DPPIV was initially synthesized as a precursor of 103 kDa, which was rapidly converted to a mature form of 109 kDa (Figure 4A). The conversion of the 103-kDa form to the 109-kDa form is due to the processing of its N-linked oligosaccharides from the high-mannose type to the complex type, as demonstrated by treatment with endoglycosidase H (Figure 4B). In fact, the entire sequence of DPPIV contains eight potential sites for glycosylation (Ogata et al., 1989).

In hepatocytes from 344/CRJ, a newly synthesized protein was also immunoprecipitated with anti-DPPIV IgG and determined to have the same molecular mass (103 kDa) as the wild-type precursor (Figure 4C). In contrast to the wild-type DPPIV, however, the mutant precursor was not processed into the mature form but rapidly disappeared along with chase times. The finding that no radioactive form was detectable even in the medium rules out the possibility that the mutant DPPIV would be converted to a secretory form. Treatment with endoglycosidase H confirmed that the mutant molecule contains only the high-mannose-type oligosaccharides (Figure

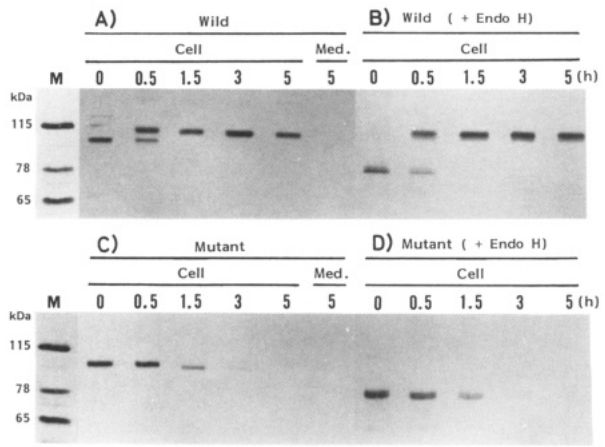

FIGURE 4: Rapid degradation of the mutant DPPIV in cultured hepatocytes. Hepatocytes from the wild (panels A and B) mutant (C and D) rats were pulse-labeled for 20 min with [^{35}S]methionine and then chased. At the indicated times of chase, cells were separated from the medium (Med.) and lysed, followed by immunoprecipitation of DPPIV. The immunoprecipitates before (A and C) and after (B and D) treatment with endoglycosidase H were analyzed by SDS–PAGE (9% gels) and fluorography. Lane M, molecular mass markers.

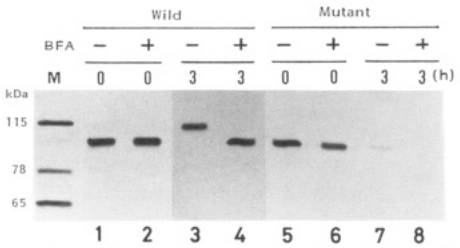

FIGURE 5: Effect of brefeldin A on the degradation of DPPIV in cultured hepatocytes. Hepatocytes from the wild and mutant rats were preincubated at 37 °C for 30 min in the absence (–) or presence (+) of brefeldin A (5 μg/mL). The cells were then labeled with [^{35}S]methionine for 20 min and chased in the absence or presence of the drug, as indicated. At zero time and 3 h of chase, cell lysates were prepared and subjected to immunoprecipitation with anti-DPPIV IgG. The immunoprecipitates were analyzed by SDS–PAGE and fluorography. Lane M, molecular mass markers.

4D). These results suggest that the mutant DPPIV is actually synthesized as the immature form but it is rapidly degraded without being processed into the mature form. No maturation of its oligosaccharides into the complex type suggests that the degradation occurs within the ER and/or early Golgi compartment. The fungal metabolite brefeldin A, a potent inhibitor of protein transport from ER to Golgi, is known to cause an accumulation of exportable proteins in the ER (Misumi et al., 1986; Fujiwara et al., 1988; Takami et al., 1990). When cells were treated with brefeldin A, the mutant DPPIV was degraded as rapidly as in the nontreated cells (Figure 5, lanes 7 and 8); a small difference observed between the two lanes was found to be due to sample loading (see Figure 6). The wild-type DPPIV, though remaining as the immature form even after 3 h of chase, was not degraded in the presence of the drug (Figure 5, lane 4). The fluorograms obtained by these experiments were densitometrically scanned, and relative densities of the wild-type and mutant DPPIVs were plotted as percentages in a log scale (Figure 6). The degradation of the mutant form follows first-order kinetics with a half-life of 54 min, whereas the wild-type is found to have a half-life of 38 h.

Confirmation of the Mutation Gly^{633} → Arg Responsible for DPPIV Degradation. We then constructed expression plasmids encoding the wild-type and mutant DPPIVs (pSD and pSDM, respectively). Two other plasmids, pSD/GR

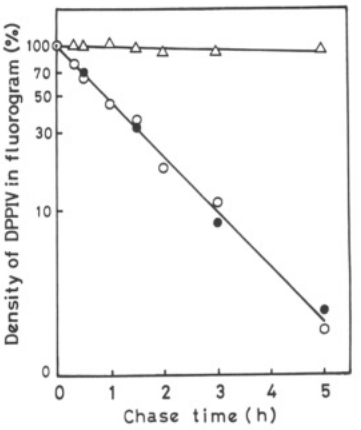

FIGURE 6: Degradation kinetics of the wild-type and mutant DPPIVs. Hepatocytes from the wild-type (△) and mutant (○, ●) rats were pulse-labeled with [^{35}S]methionine for 20 min and chased in the absence (△, ○) or presence (●) of brefeldin A (5 μg/mL). Immunoprecipitates of cell lysates obtained at the indicated times of chase were analyzed by SDS–PAGE, as shown in Figures 4 and 5. The resulting fluorograms were densitometrically scanned. Relative densities were determined by taking the density of each band at zero time of chase as 100%, and are plotted in a log scale. Each value represents the mean of three separate experiments.

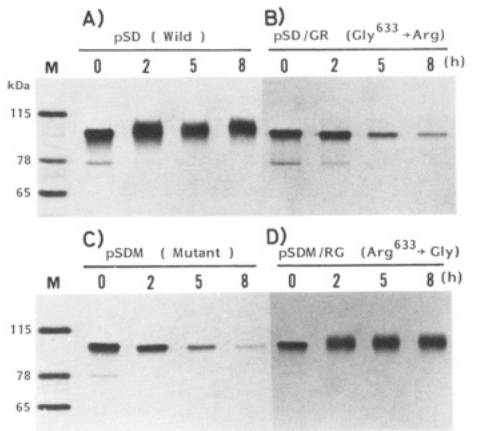

FIGURE 7: Degradation of DPPIVs expressed in the transfected cells. COS-1 cells were transfected with the indicated plasmids: (A) pSD (wild); (B) pSD/GR (Gly^{633} → Arg); (C) pSDM (mutant); (D) pSDM/RG (Arg^{633} → Gly). The cells were labeled with [^{35}S]-methionine for 20 min and then chased. At the indicated times of chase, cell lysates were prepared and subjected to immunoprecipitation with anti-DPPIV IgG. The immunoprecipitates were analyzed by SDS–PAGE and fluorography. Lane M, molecular mass markers.

(Gly^{633} → Arg) and pSDM/RG (Arg^{633} → Gly), were also prepared by site-directed mutagenesis of pSD and pSDM, respectively. These plasmids were transfected into COS-1 cells, and the expressed DPPIVs were analyzed by pulse–chase experiments. The wild-type DPPIV synthesized in COS-1 cells (Figure 7A) showed essentially the same processing and stability as that in hepatocytes, although a processed form migrated in SDS–PAGE as a band slightly faster and broader than the mature form synthesized by hepatocytes (see Figure 4A). Such a difference in molecular mass may be due to a difference in the extent of its glycosylation between the two cells (Misumi et al., 1990; Ogata et al., 1992). When the plasmid pSD/GR (Gly^{633} → Arg) was transfected, the protein expressed was never processed to a larger molecule and was rapidly degraded (Figure 7B), as seen for the mutant DPPIV in hepatocytes. The same event was observed for DPPIV expressed by transfection with the original mutant plasmid pSDM (Figure 7C), as expected. Finally, the revertant plasmid pSDM/RG (Arg^{633} → Gly) directed the synthesis of DPPIV which had the same stability (Figure 7D)

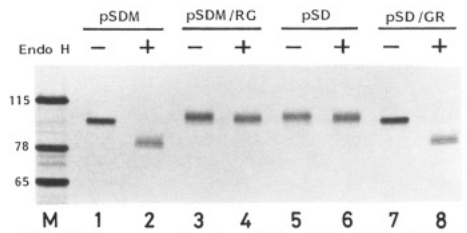

FIGURE 8: Sensitivity to endoglycosidase H of the DPPIV forms expressed in the transfected cells. ^{35}S-Labeled DPPIVs were prepared from COS-1 cells which had been transfected with the indicated plasmids, labeled with [^{35}S]methionine for 20 min, and then chased for 5 h, as shown in Figure 7. The samples were incubated at 37 °C for 16 h in 50 mM acetate buffer (pH 5.5) in the absence (–) or presence (+) of endoglycosidase H (0.2 unit/mL), and subjected to SDS–PAGE and fluorography. The samples in lanes 3–6 were adjusted to contain about 3000 dpm/lane, while those in other lanes contained total radioactivities obtained. Lane M, molecular mass markers.

Table I: Enzyme Activities of DPPIV in the Transfected Cells[a]

plasmid	mutation (position 633)	DPPIV act. [μmol min^{-1} (mg of protein)$^{-1}$]
mock		0.002 ± 0.001
pSD (wild)		0.498 ± 0.045
pSD/GR	Gly → Arg	0.007 ± 0.002
pSDM (mutant)	Gly → Arg	0.009 ± 0.002
pSDM/RG	Arg → Gly	0.452 ± 0.039

[a] COS-1 cells were transfected with the indicated plasmids and cultured for 2 days. The cells were harvested, lysed, and subjected to determination of DPPIV activity with Gly-Pro-*p*-nitroaniline as a substrate. Values represent the means ± SE of three separate experiments.

as that of the wild-type DPPIV. In addition, all the degradable mutants were found to be sensitive to endoglycosidase H treatment even after a prolonged time of chase (Figure 8). Taken together, these results indicate that the single substitution of Gly^{633} → Arg is sufficient to cause the retention and rapid degradation of DPPIV, possibly, in the ER.

It was also confirmed that no significant increase of the enzyme activity was detectable in the cells transfected with either of the mutant plasmids (Gly^{633} → Arg), in contrast to its remarkable increase in the cells expressing the wild-type or revertant DPPIV (Table I).

Immunoelectron Microscopy. Figure 9 shows immunoelectron-microscopic profiles of COS-1 cells which were transfected with the wild-type plasmid pSD (A) and with the mutant pSDM (B). The mutant DPPIV expressed was detectable only in the ER and nuclear envelope but not in the Golgi complex and on the cell surface. This is in contrast to the staining profile of the wild-type DPPIV which was concentrated in the Golgi complex as well as on the surface. These observations support the biochemical data obtained in this study.

DISCUSSION

Cloning and sequence analysis of the cDNA revealed the single substitution of Gly^{633} → Arg in DPPIV of 344/CRJ caused by a point mutation. The residue Gly^{633} is found in the active-site sequence Gly^{629}-Trp-Ser-Tyr-Gly^{633} of DPPIV which corresponds to the consensus sequence Gly-X-Ser-X-Gly proposed for the active site of serine hydrolases (Brenner, 1988). Our previous study (Ogata et al., 1992) demonstrated that in DPPIV, any single substitution of the residues in the active-site sequence except for Trp^{630} results in the complete loss of enzyme activity. Thus, no enzyme activity of DPPIV in any tissues of 344/CRJ is easily explained by the substitution of Gly^{633} → Arg in the mutant DPPIV.

In hepatocytes, DPPIV was initially synthesized as the 103-kDa form and rapidly converted to the mature form of 109

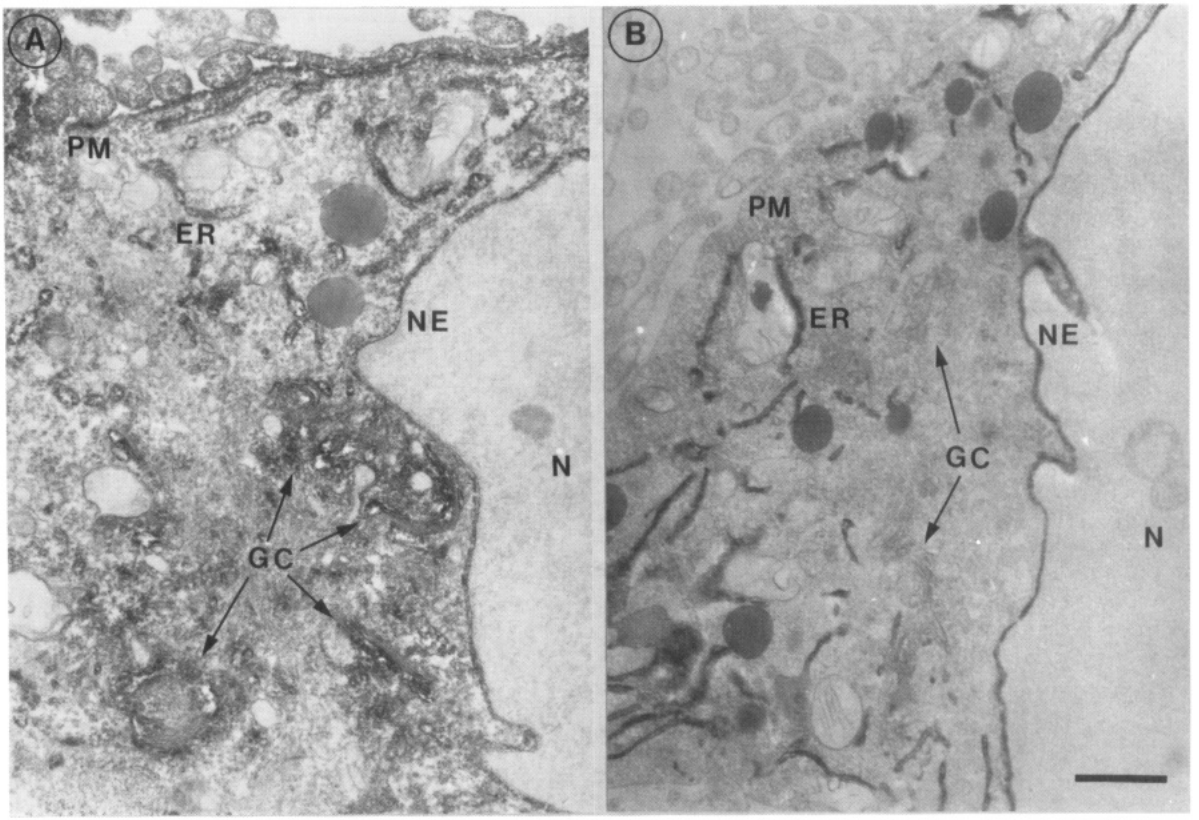

FIGURE 9: Immunoelectron microscopy of the transfected cells. COS-1 cells were transfected with the wild-type plasmid pSD (A) and with the mutant pSDM (B) and cultured for 2 days. The cells were fixed, permeabilized, and subjected to staining of DPPIV with the immunoperoxidase. ER, endoplasmic reticulum; GC, Golgi complex; N, nucleus; NE, nuclear envelope; PM, plasma membrane. Bar, 1 μm.

kDa by processing of its oligosaccharides from the high-mannose type to the complex type which occurs in the Golgi complex (Kornfeld & Kornfeld, 1985). The mutant DPPIV was also synthesized as the 103-kDa form, which was, however, rapidly degraded ($t_{1/2}$ = about 50 min) without being processed into the mature 109-kDa form. The mutant DPPIV remaining as the 103-kDa form was confirmed to have the high-mannose-type oligosaccharides. In addition, this degradation could occur in the absence of anterograde transport from the ER to the cell surface, as demonstrated by the brefeldin A experiments. Thus, these results suggest that the mutant DPPIV is retained in the ER followed by its rapid degradation therein, although the possibility that the pre-Golgi and/or early Golgi compartments are involved in the event cannot be completely ruled out at present. The results also account for the reason why the mutant DPPIV was not detectable in the plasma membrane even by immunochemical methods.

It is quite unusual that the retention and rapid degradation of DPPIV in the ER are caused by the single substitution of $Gly^{633} \rightarrow Arg$ at the active-site sequence. Other possibilities are also considered that hepatocytes as well as other cells in the 344/CRJ tissues have a primary defect in the transport system from ER to Golgi, and/or produce some protease(s) involved in degradation of particular proteins such as DPPIV. In the hepatocytes of 344/CRJ, however, all other ectoenzymes examined, such as alkaline phosphatase, 5′-nucleotidase, and γ-glutamyl transpeptidase, were stably expressed on the cell surface, and newly synthesized secretory proteins including albumin and α_1-protease inhibitor were secreted with the same kinetics as those in hepatocytes from other rats (data not shown). Finally, the conclusive evidence was obtained by transfection/expression experiments in COS-1 cells. Only the mutant DPPIV with substitution of $Gly^{633} \rightarrow Arg$, either naturally occurring or being constructed by site-directed mutagenesis, was rapidly degraded in the transfected COS-1 cells as observed in the hepatocytes. These results clearly indicate that the substitution of $Gly^{633} \rightarrow Arg$ is sufficient to cause the drastic change in intracellular transport and stability of DPPIV. Immunoelectron-microscopic observations of the transfected cells are also favorable to our conclusion that the mutant DPPIV is retained and degraded in the ER.

It has become apparent that many membrane proteins which are normally transported from the ER to the cell surface, when improperly folded or incompletely assembled, are retained in the ER (Lodish, 1988; Hurtley & Helenius, 1989). The abnormal or unassembled polypeptide subunits, for example, the α subunit of the T-cell antigen receptor (Lippincott-Schwartz et al., 1988; Bonifacino et al., 1991) and the H2a subunit of the human asialoglycoprotein receptor (Amara et al., 1989; Wikström & Lodish, 1992), undergo rapid degradation within the ER by a proteolytic system which has not yet been fully characterized (Klausner & Sitia, 1990). It is of interest to note that the α subunit of the T-cell antigen receptor is completely degraded without detection of any proteolytic intermediate, as observed here for the mutant DPPIV, whereas the H2a subunit produces an intermediate fragment at the initial phase of degradation.

DPPIV is known to be a dimer composed of identical subunits without being covalently linked (Kenny et al., 1976; Elovson, 1980). If the substitution of $Gly^{633} \rightarrow Arg$ critically impedes the dimerization of the subunits, the retention and degradation of the mutant DPPIV would be explained as the same event observed for the other proteins mentioned above. Recent observations, however, suggested that the dimerization of the DPPIV subunits takes place in a late-Golgi compartment (Jascur et al., 1991), in contrast to the evidence that most proteins are oligomerized in the ER (Hurtley & Helenius, 1989). In that case, the subunit monomer itself would be altered by the mutation to have an abnormal conformation that is recognized as a signal for the retention and degradation in the ER. The human PiZ α_1-antitrypsin variant is considered to be a comparable example. Although α_1-antitrypsin is a monomeric secretory protein, it is likely that the substitution of $Leu^{342} \rightarrow Lys$ in the PiZ variant causes the newly synthesized protein to misfold, resulting in its degradation within the ER (Le et al., 1990, 1992). Thus, in any cases of monomer or oligomer, misfolded proteins could be subjected to retention and degradation in the ER. The possible conformational change in the mutant DPPIV caused by the substitution of $Gly^{633} \rightarrow Arg$ remains to be determined.

Studies using the DPPIV-deficient rat have provided conclusive evidence for the obligatory role of DPPIV in the renal handling of proline (and hydroxyproline)-containing peptides (Tiruppathi et al., 1990a) and for the presence of an electrogenic tripeptide-proton symport in renal brush-border membranes (Tiruppathi et al., 1990b). On the other hand, recent observations suggest that DPPIV, in addition to its peptidase activity, may play a role in fibronectin-mediated interactions of cells with the extracellular matrix (Piazza et al., 1989) and in activation of T lymphocytes (Marquet et al., 1992). Such particular functions proposed for DPPIV will also be specified by use of cells derived from the DPPIV-dificient rat.

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