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Domain-specific N-glycosylation of the membrane glycoprotein dipeptidylpeptidase IV (CD26) influences its subcellular trafficking, biological stability, enzyme activity and protein folding

Hua FAN, Wenmao MENG, Christiane KILIAN, Sabine GRAMS and Werner REUTTER
Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Germany

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Dipeptidyl peptidase IV (DPPIV, CD26) is an N-glycosylated type II plasma membrane protein. The primary structure of rat wild-type DPPIV contains eight potential N-glycosylation sites. To investigate the role of N-glycosylation in the function of DPPIV, three of its asparagine residues were separately converted to glutamine by site-directed mutagenesis. The resulting N-glycosylation mutants of rat DPPIV were studied in stable transfected Chinese hamster ovary cells. All three N-glycosylation mutants of DPPIV showed a reduced half-life, as well as differing degrees of inhibition of the processing of their N-glycans. Mutation of the first (Asn83→Gln) or eighth (Asn686→Gln) N-glycosylation site had only a small effect on its enzymatic activity, cell-surface expression and dimer formation, whereas the mutation of the sixth N-glycosylation site (Asn319→Gln) abolished the enzymatic activity, eliminated cell-surface expression and prevented the dimerization of the DPPIV protein. The mutant [Gln319]DPPIV is retained in the cytoplasm and its degration was drastically increased. Our data suggest that the N-glycosylation at Asn319 is involved in protein trafficking and correct protein folding.

Keywords: N-glycosylation; dipeptidylpeptidase IV; protein stability; protein trafficking; site-directed mutagenesis.

Although most membrane proteins in nature are glycosylated, little is understood about the role of carbohydrates in protein structure, function and dynamics. Recent results suggest that N-linked oligosaccharides may play multiple roles in development, growth, function and survival of an organism. Carbohydrate units can modulate the physicochemical and biological properties of their parent proteins, such as protein folding, stability, targeting, and dynamics, as well as cell–matrix and cell–cell interactions (Kobata, 1992; Varki, 1993; Lis and Sharon, 1993; Helenius, 1994; Fiedler and Simons, 1995).

Dipeptidylpeptidase IV (DPPIV, CD26), is a widely distributed, multi-functional, highly glycosylated membrane protein (Fleischer, 1995). It cleaves Xaa-Pro dipeptides from the NH_2-terminus of peptides, and is essential for the intestinal and renal transport of proline-containing peptides (Reutter et al., 1989; Brandsch et al., 1995). Expression of DPPIV is tightly associated with cell differentiation and cell activation. It is involved in T cell activation (Fleischer, 1994; Morimoto and Schlossman, 1994; Ansorge et al., 1995).

The purified enzyme is a homodimer with non-covalently linked subunits (Elovson, 1980). The oligosaccharide side chains account for about 30% of the total protein molecular mass (Yamashita et al., 1988). Rat DPPIV contains eight potential N-glycosylation sites, whereas O-glycosylation of this protein has not been detected (Petell et al., 1987; Hong and Doyle, 1987; Ogata et al., 1989). However, the biological role of DPPIV N-glycosylation remains obscure.

In transfected Chinese hamster ovary (CHO) cells, DPPIV is initially synthesized as a 100-kDa monomer, which cotranslationally acquires eight mannose-rich N-glycosyl chains in the endoplasmic reticulum (ER). During passage through the Golgi apparatus, these are converted into complex oligosaccharides containing galactose and sialic acid, thereby increasing the size of the monomer to 110 kDa (Hong et al., 1989). In the Golgi apparatus the monomers associate with each other and give rise to homodimers of 210 kDa (Jascur et al., 1991). We have previously studied the N-glycosylation and oligosaccharide processing of DPPIV using glycosylation inhibitors. Following inhibition of primary N-glycosylation by tunicamycin, the biological stability of N-glycosylated DPPIV was dramatically reduced, and this protein could not be detected at the cell surface. However, inhibition of processing by 1-deoxymannojirimycin led to the formation of DPPIV molecules containing N-glycans of the oligomannosidic type, but did not affect the activity of the enzyme, its stability and cell-surface transport (Loch et al., 1992).

In inhibitor-treated cells, nonspecific inhibition of glycosylation of many other proteins may complicate the interpretation of

Correspondence to H. Fan, Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Arnimallee 22, D-14195 Berlin-Dahlem, Germany
Phone: +49 030 838 62 44.
Fax: +49 030 838 21 41.
E-mail: fanzheng@zedat.fu-berlin.de

Abbreviations. DPPIV, dipeptidylpeptidase IV; CHO, Chinese hamster ovary; [Gln83]DPPIV, etc., DPPIV with Asn83 replaced by Gln etc.; NaCl/P_i, phosphate-buffered saline; endo-H, endo-β-*N*-acetylglucosaminidase H; FITC, fluorescein isothiocyanate; CMV, cytomegalovirus; Gly-Pro-NHNp, glycyl-prolyl *p*-nitroanilide.

Enzymes. Dipeptidyl peptidase IV (EC 3.4.14.5); endoglycosidase H, endo-β-*N*-acetylglucosaminidase H (EC 3.2.1.96); acetylcholinesterase (EC 3.1.1.7).

the results. To investigate the biological function of single N-glycosylations of DPPIV under physiological conditions, site-directed mutagenesis was therefore used in the present work to abolish the addition of single oligosaccharides to specific glycosylation sites.

The extracellular domain of DPPIV, including all eight putative N-glycosylation sites, can be further divided into three regions with prominent structural features. First, adjacent to the transmembrane domain, is a region harboring a cluster of five out of eight putative N-glycosylation sites. This is followed by a central cysteine-rich region expressing nine of the twelve cysteine residues which probably form disulfide bridges. Third, the C-terminus itself includes the active site of the serine protease (Reutter et al., 1995) (Fig. 1). In this work, three N-glycosylation sites were chosen for mutation, each from one of three extracellular domains, to investigate the influence of these domains on the biological functions and biochemical properties of the protein. Wild-type DPPIV and its individual N-glycosylation mutants were expressed in CHO cells lacking endogenous DPPIV; stable transfectants were cloned and analysed biochemically as well as immunochemically. In this report we demonstrate that defective glycosylation of DPPIV at single specific sites in the molecule has various effects on the biochemical properties and functions of this protein.

EXPERIMENTAL PROCEDURES

Materials. *Escherichia coli*, strains HB101, XL1 Blue and the plasmid pBluescript II SK were obtained from Stratagene. Expression plasmid pRC/CMV was obtained from In vitrogen BV. Restriction endonucleases, T4-DNA ligase and the random primed DNA labeling kit were either from Gibco BRL or Boehringer Mannheim. The T7 sequencing kit was from Pharmacia LKB Biotechnology Inc. Site-directed-mutagenesis kit, nitrocellulose and nylon membranes were from Amersham. [^{35}S]Methionine (800 Ci/mmol), [^{35}S]dATP [αS] (1200 Ci/mmol) and [α-^{32}P]dATP (3000 Ci/mmol) were purchased from DuPont. Bicinchoninic acid protein assay reagent was from Pierce. Endoglycosidase H (endo H) and protein-A−Sepharose 4B were from Boehringer Mannheim. Glycyl-prolyl *p*-nitroanilide (Gly-Pro-NHNp) was from Bachem. Minimum essential medium and fetal bovine serum were from Gibco BRL. Dulbecco's modified essential medium without glutamine, methionine and cysteine was obtained from ICN. Immunofluorescence-conjugated second antibodies, as well as other reagents and chemicals were from Sigma. Antiserum to wild-type DPPIV and mAb 13.4 were prepared as described previously (Becker et al., 1986; Hartel-Schenk et al., 1991). The Mac Molly sequence analysis software program was a gift of Dr Burghardt Wittig in this institute.

Construction of DPPIV mutants lacking single N-glycans by site-directed mutagenesis. Wild-type DPPIV cDNA *Hin*dIII−*Bgl*II fragment (position −35 to 280), *Pst*I−*Pst*I fragment (734−1074) and *Sph*I−*Bgl*II fragment (1780−2156) were cut from pBSK/DPPIV, a plasmid containing the entire wild-type DPPIV cDNA. The three fragments including the first (Asn83), sixth (Asn319) and eighth (Asn686) N-glycosylation sites, respectively, were each inserted into *Hin*dIII−*Bam*HI, *Pst*I−*Pst*I and *Sph*I−*Bam*HI sites of M13mp19, M13mp18 and M13mp19, respectively, and confirmed by restriction endonuclease mapping. The sense strands of these M13 derivatives were used as templates for site-directed mutagenesis. Three synthetic antisense oligonucleotides, 5′-pCCAAGAAAATGGAGCTTTGCCCGTGTTCAGC-3′ (31 residues), 5′-pCGCCATCACGGAATATTGCTGAATCCTCCTGAGCC-3′ (35 residues) and 5′-pGACTGTTGATTGCCTGTAATGG-3′ (22 residues) were used to replace the codons Asn83, Asn319 and Asn686 by Gln codons (underlined), respectively. The reaction was performed using the Amersham M13-phage system according to the manufacturer's protocol. After site-directed mutagenesis, individual mutations were confirmed by sequencing according to the method of Sanger et al. (1977). Mutation-carrying DPPIV inserts were then isolated from M13 derivatives and inserted into pBSK/DPPIV DNA in exchange for the wild-type fragments using *Hin*dIII and *Sau*3A, *Pst*I, and *Sph*I and *Sau*3A boundaries, respectively.

Plasmid construction for expression of wild-type DPPIV, [Gln83]DPPIV, [Gln319]DPPIV and [Gln686]DPPIV. The *Hin*dIII−*Apa*I cDNA fragments containing the full coding sequences for wild-type DPPIV, [Gln83]DPPIV, [Gln319]DPPIV and [Gln686]DPPIV were isolated by double-digestion with *Hin*dIII and *Apa*I, and integrated between *Hin*dIII and *Apa*I sites of the expression plasmid pRC/CMV. Each construction was confirmed by restriction endonuclease mapping and sequencing.

Transfection of CHO cells and selection of stable transfectants. Each plasmid DNA (20 μg) was transfected into 5×10^6 CHO cells by electroporation (Gene Pulser, Bio-Rad) as described previously (Oda et al., 1989). The transfected cells were cultured in a minimum essential α medium containing 440 mg/l glutamine and 10% fetal calf serum in 10-cm dishes for 48 h, then selected with 400 mg/l geneticin G418 in the same medium for the following two to three weeks. The surviving cells were subsequently cloned and further incubated with selection medium.

Analysis of expressed mRNAs by northern blotting. Total RNA was prepared from each transfected clone according to the RNAzol™ B method (Chomczynski and Sacchi, 1987). 20 μg of each sample were denatured with 50% formamide and 6.5% formaldehyde, and the RNAs were electrophoretically separated in 1.5% agarose gels containing 2.2 M formaldehyde. RNAs of the gel were transferred onto a nylon membrane and hybridized with ^{32}P-labeled *Bgl*II−*Bgl*II (position 280−2156) or *Pst*I−*Pst*I (position 734−1074) gene probes isolated from pBSK/DPPIV, which had been prepared using a random-primed DNA labeling procedure (Feinberg and Vogelstein, 1983). Hybridization was performed as described in the manufacturer's protocol (Sambrook et al., 1989), and mRNA species were detected by autoradiography.

Immunoprecipitation and western blotting analysis. Cells were harvested after treatment with NaCl/P_i containing 0.05% (mass/vol.) EDTA for 5 min. After sedimentation, the cell pellets were solubilized at 4°C for 1 h with NaCl/P_i containing 1% (mass/vol.) Triton X-100. Following centrifugation at 48000 *g* for 30 min, aliquots of the supernatants were subjected to western blotting. For analysis under denaturating conditions, samples of supernatants were incubated with sample buffer [60 mM Tris/HCl pH 6.8, 5% 2-mercaptopropandiol, 0.003% bromophenol blue, 10% (by vol.) glycerol and 3% (mass/vol.) SDS] and boiled for 5 min. For analysis under non-denaturating conditions, the samples were only incubated with non-denaturating sample buffer [60 mM Tris/HCl pH 6.8, 0.003% bromophenol blue, 10% (by vol.) glycerol and 0.5% (mass/vol.) SDS] and not boiled.

For immunoprecipitation, the anti-DPPIV mAb 13.4 was incubated with protein-A−Sepharose at 4°C for 2 h before the cell supernatants were incubated overnight with mAb 13.4 conjugated to protein-A−Sepharose at 4°C. After extensive washing, immunoprecipitates were eluted by boiling for 4 min in SDS sample buffer for electrophoresis. SDS/PAGE was performed according to Laemmli (1970). After electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane. Polyclonal antiserum against wild-type DPPIV or mAb 13.4 was

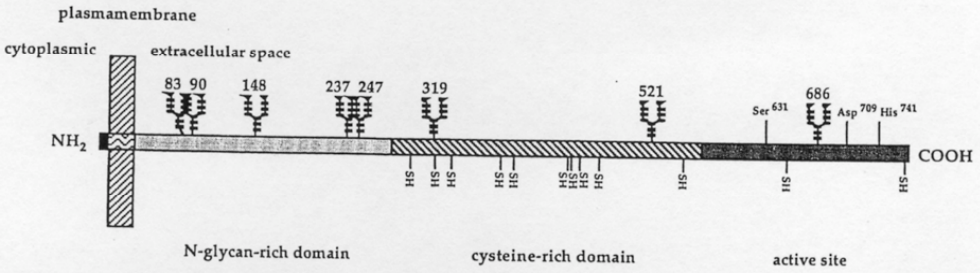

Fig. 1. Schematic representation of N-glycosylation sites and domains of wild-type DPPIV deduced from its primary structure. The Y-shaped structures represent N-glycans; SH, cysteine residue.

used for immunostaining; visualisation was performed with peroxidase-conjugated pig anti-rabbit or rabbit anti-mouse IgG (Sigma) and the chemiluminescent reagent, luminol.

Immunoflurescence microscopy. Cells were incubated in NaCl/P_i, then fixed with 3% (by vol.) formaldehyde in NaCl/P_i at room temperature for 10 min (if indicated, after permeabilization with 0.1% Triton X-100 in NaCl/P_i at room temperature for 5 min). Cells were then extensively washed with NaCl/P_i and blocked with 0.1 M glycine in NaCl/P_i for 30 min and washed with NaCl/P_i again. Polyclonal antibodies against wild-type DPPIV and mAb 13.4 were used for immunostaining at room temperature for 2 h. After further washing with NaCl/P_i, the cells were incubated with fluorescein-isothiocyanate conjugated rabbit anti-mouse IgG (diluted 1:200) at room temperature for 1h. After extensive washing with NaCl/P_i, the cells were mounted with glycerol/NaCl/P_i (10:1, by vol.) for fluorescence microscopy.

Pulse-chase experiments. Cells (2×10^6 cells/dish) were incubated at 37°C for 4 h in Dulbecco's modified essential medium lacking cystine and methionine. The cells were pulse-labeled with [^{35}S]methionine, for 1 h with 100 µCi/dish. After the chase times indicated, cell lysates were prepared, immunoprecipitated with anti-DPPIV mAb 13.4 and subjected to SDS/PAGE (7.5%) followed by scanning phosphoimaging (Phosphoimager™ 445 S1, Molecular Dynamics).

Determination of enzymatic activity of DPPIV mutants. The pellets obtained from transfected cells were solubilised at 4°C for 1 h with NaCl/P_i containing 1% (mass/vol.) Triton X-100 and 1 mM EDTA, and subjected to enzyme activity assay. DPPIV enzymatic activity was determined with Gly-Pro-NHNp as substrate (Nagatsu et al., 1976). The relative DPPIV specific activity was calculated by comparing the activity of the same amount of protein from wild-type DPPIV and DPPIV mutants, which was determined by scanning the immunoblot after loading with identical amounts of total solubilised protein (data not show).

Endoglycosidase H treatment. Immunoprecipitates were eluted by boiling for 4 min in buffer containing 0.4% SDS, 1% 2-mercaptopropanediol and 40 mM EDTA. Endoglycosidase H (endo H) treatment was performed with endo H (0.02 U/80 ml) at 37°C for 16 h in 50 mM sodium acetate pH 5.5.

Other methods. The protein concentration in the supernatant was determined using the bicinchoninic acid protein assay reagent. DNA sequencing as performed by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using the T7-sequencing™ kit (Pharmacia LKB).

RESULTS

Generation of Asn→Gln DPPIV N-glycosylation mutations by site-directed mutagenesis. Three N-glycosylation sites were selected for conversion to glutamine by site-directed mutagenesis. The first is located at position Asn83 near the membrane-anchoring domain. The sixth is located at position Asn319 within the central Cys-rich region, while the eighth is located at position Asn686 close to the enzyme active centre in the C-terminus of the primary structure (Fig. 1). The strategy of DPPIV subconstructs and oligonucleotide-controlled mutagenesis is described in Experimental Procedures. The correct mutations were confirmed by sequencing. The resulting three DPPIV N-glycosylation mutants were designated as [Gln83]DPPIV, [Gln319]DPPIV and [Gln686]DPPIV.

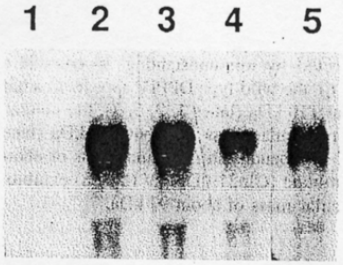

Fig. 2. Northern blot analysis of DPPIV mRNAs in transfected CHO cells. Total RNA was prepared from transfected clones using the RNAzol™ B method. 20 µg of each preparation were denatured and separated by electrophoresis on a 1.5% agarose gel. Separated RNA was transferred to a nylon membrane and hybridized with ^{32}P-labeled *BglII*–*BglII* (position 281–2156) or *PstI*–*PstI* (position 735–1074) fragments as prepared from pBSK/DPPIV. RNA size markers are 28S and 18S rRNAs of CHO cells. DPPIV mRNA could not be detected in control CHO cells prior to transfection (lane 1), whereas the mRNA of wild-type DPPIV (lane 2), [Gln83]DPPIV (lane 3), [Gln319]DPPIV (lane 4) and [Gln686]DPPIV (lane 5) were present in transfected CHO clones.

Expression of wild-type and mutant DPPIV in transfected CHO cells. In order to characterize the wild-type DPPIV and its mutants, CHO cells were transfected with their cDNAs. CHO cells were chosen because they do not express endogenous DPPIV. The coding sequences for wild-type and mutant DPPIV were integrated into the pRC/CMV expression vector separately; they were then under the transcriptional control of the enhancer/promoter sequence of human cytomegalovirus (CMV). Stable transfectants were selected and cloned via geneticin selection.

mRNA expression of wild-type and mutant DPPIV in transfected CHO cells. In order to determine the mRNA expression of transfected wild-type and mutant DPPIV in CHO cells, northern blot analysis was performed to detect DPPIV mRNA. Fig. 2 shows that DPPIV mRNA could not be detected in non-transfected control CHO cells (lane 1), whereas the

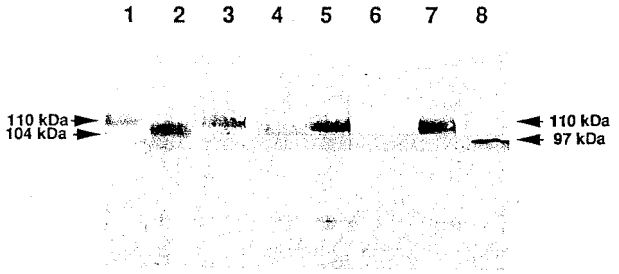

Fig. 3. Immunoprecipitation and western blot analysis of wild-type and mutant DPPIV in transfected CHO cells. The pellets obtained from transfected cells were solubilised at 4 °C for 1 h with NaCl/P_i containing 1% (mass/vol.) Triton X-100 and 1 mM EDTA. 300 μg total protein in solution were subjected to immunoprecipitation with anti-DPPIV mAb 13.4. The immunoprecipitates were analysed by SDS/PAGE (7.5%) and detected by immunostaining. In contrast to untransfected CHO cells (lane 6), the wild-type DPPIV appears as a band with molecular mass of about 110 kDa (lanes 1, 3, 5, 7), the mutant [Gln83]DPPIV as a band with molecular mass of about 104 kDa (lane 2), the mutant [Gln686]DPPIV as a band with molecular mass of about 107 kDa (lane 4), whereas the mutant [Gln319]DPPIV (lane 8) exhibits only as a sharp band with molecular mass of about 97 kDa.

mRNAs of wild-type DPPIV (lane 2), [Gln83]DPPIV (lane 3), [Gln319]DPPIV (lane 4) and [Gln686]DPPIV (lane 5) were present in transfected CHO clones.

Effects of defective N-glycosylation of DPPIV on electrophoretic mobility. Protein expression in wild-type and mutant DPPIV CHO clones was determined by immunoprecipitation and western blotting (Fig. 3). In comparison with transfected wild-type DPPIV with an apparent molecular mass of about 110 kDa (lanes 1, 3, 5, 7), most [Gln83]DPPIV (lane 2) and [Gln686]DPPIV (lane 4) mutants showed a band in this region but with slightly increased mobilities (104 kDa and 107 kDa, respectively), whereas mutant [Gln319]DPPIV (lane 8) exhibited a significantly increased mobility (97 kDa). This band could represent an incompletely glycosylated form.

N-glycosylation processing of wild-type and mutant DPPIV in transfected CHO cells. In order to gain insight into the processing of carbohydrate residues of wild-type and mutant DPPIV, we performed a pulse/chase experiment with transfected clones and digested the immunoprecipitated peptides with endo-H. Glycoproteins containing only mannose-rich glycans are endo-H-sensitive, whereas hybrid or mature complexes form are endo-H-resistant.

In this experiment cells were pulse-labeled for 30 min with [^{35}S]methionine, then chased with excess unlabeled methionine for various times. After immunoprecipitation with mAb 13.4, half of the immunoprecipitates were subjected to treatment with endo H for 16 h at 37 °C. Fig. 4 shows that, after a 30-min pulse, about 50% of the oligosaccharides of wild-type DPPIV had been processed to a mature complex form, whereas the corresponding values for [Gln83]DPPIV and [Gln686]DPPIV are about 25% and 40%, respectively. After a 60-min chase almost all of the oligosaccharides of wild-type DPPIV were processed to mature complex form, while the processing of [Gln83]DPPIV reached about 50% and [Gln686]DPPIV about 75%. This result suggests that N-glycosylation mutations at positions Asn83 and Asn686 may retard the N-glycosylation processing of this protein. In contrast, most of the [Gln319]DPPIV was converted into a polypeptide of apparent molecular mass 84 kDa after endo H digestion at each time, indicating that most mutant proteins of

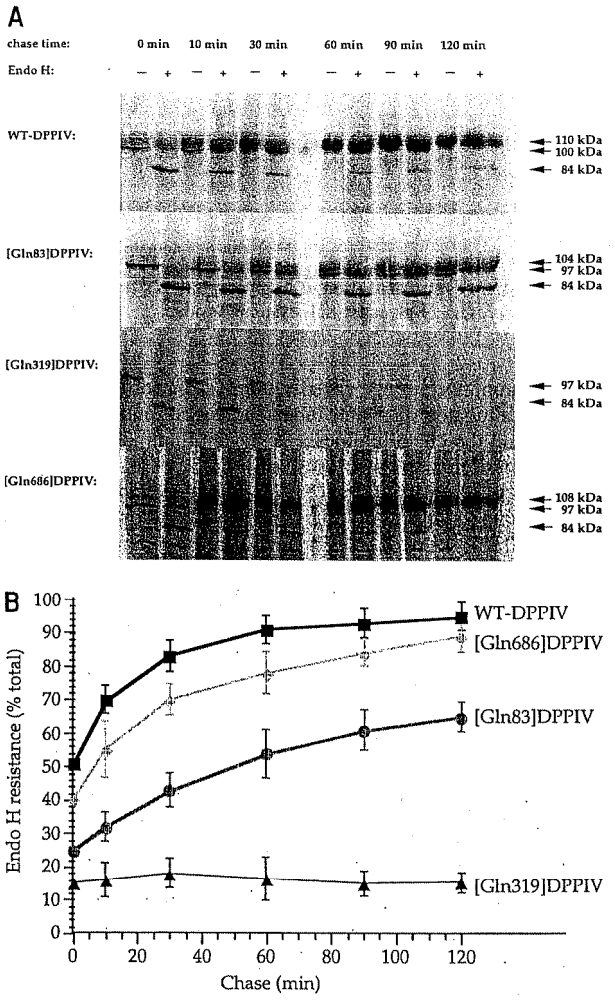

Fig. 4. Analysis of the processing of wild-type and mutant DPPIV in transfected CHO cells. (A) CHO/wild-type DPPIV, CHO/[Gln83]DPPIV, CHO/[Gln319]DPPIV and CHO/[Gln686]DPPIV were pulse-labeled with [^{35}S]methionine for 30 min and chased for 0 min, 10 min, 30 min, 60 min, 90 min or 120 min. Immunoprecipitates of cell lysates obtained at the indicated times of chase were treated with endo H (0.02 U/80 ml) at 37 °C for 16 h in 50 mM sodium acetate pH 5.5 and analysed by SDS/PAGE. As indicated, after digestion with endo H, the mannose-rich form of DPPIV and its mutants were converted into a polypeptide of apparent molecular mass 84-kDa. The lanes in the middle of the SDS/PAGEs were used for a non-radioactive marker. (B) The protein bands obtained in puls/chase experiments were analysed by phosphoimager scanning. Each value indicated represents the average of three separate experiments. The total amounts of both endo-H-resistant and endo-H-sensitive protein band at each time were set as 100%.

[Gln319]DPPIV were not processed to a mature complex form within the tested time period.

Half-life of N-glycosylation mutants of DPPIV in transfected CHO cells. In order to study the effect of the N-glycosylation on protein stability, the half-life of the DPPIV mutants was determined by pulse/chase experiments. Transfected CHO clones were pulse-labeled for 1 h with [^{35}S]methionine, then chased with an excess of unlabeled Met for various times. Following termination of the reaction, wild-type and mutant DPPIV were immunoprecipitated. The immunoprecipitates were resolved by

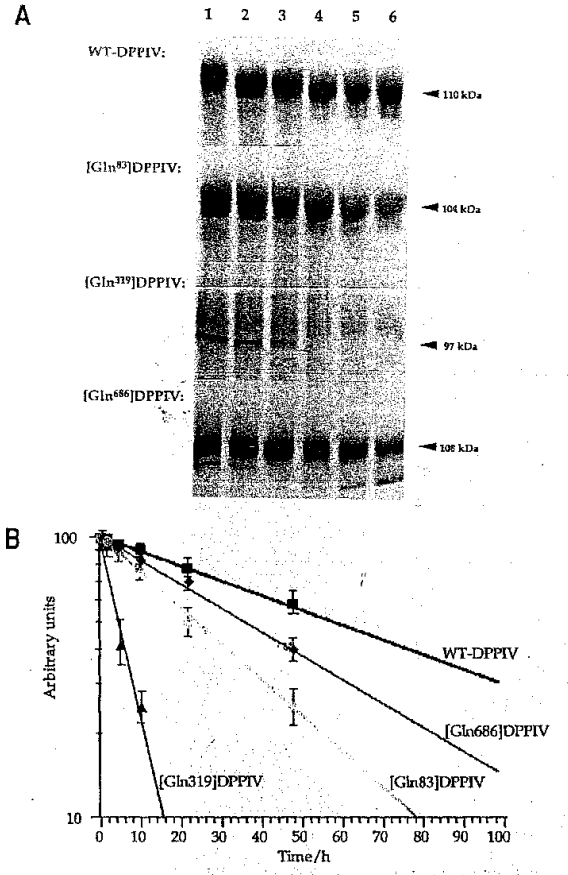

Fig. 5. Biological stability of wild-type and mutant DPPIV in transfected CHO cells. (A) CHO/wild-type DPPIV, CHO/[Gln83]-DPPIV, CHO/[Gln319]DPPIV and CHO/[Gln686]DPPIV were pulse-labeled with [^{35}S]methionine for 1 h and chased for 0 min (lane 1), 2 h (lane 2), 5 h (lane 3), 10 h (lane 4), 22 h (lane 5) or 44 h (lane 6). Immunoprecipitates of cell lysates obtained at the indicated times of chase were analysed by SDS/PAGE. (B) The results of the pulse/chase experiments were analysed by phosphoimager scanning. Each time point represents the average value derived from three separate experiments. The radioactivities obtained by immunoprecipitation of the pulse-labeled cells without chase were set as 100%. All other values were expressed relative to this value.

SDS/PAGE and detected and quantified by phosphoimaging. As shown in Fig. 5, wild-type DPPIV is very stable with a half-life of about 50 h. In contrast, the biological stabilities of the three N-glycosylation mutants of DPPIV were significantly reduced, but to different extents. [Gln83]DPPIV possessed an average half-life of about 25 h and [Gln686]DPPIV of about 35 h. The half-life of [Gln319]DPPIV was remarkable reduced to 4 h, which is more than 10 times shorter than that of wild-type DPPIV.

Enzymatic activity of the N-glycosylation mutants of DPPIV. The enzymatic activity of N-glycosylation mutants was compared with that of wild-type DPPIV. The relative DPPIV specific activity was calculated by comparing the activities of equal amounts of protein from the wild-type and mutants. Fig. 6 shows that the specific enzymatic activity of [Gln83]DPPIV (lane 3) and [Gln686]DPPIV (lane 5) were only slightly reduced in comparison with wild-type DPPIV (lane 2), whereas [Gln319]-DPPIV (lane 4) had no measurable enzymatic activity.

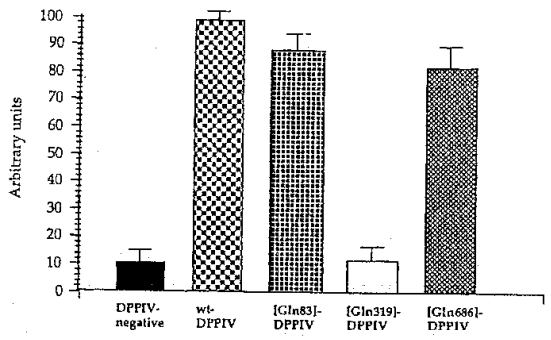

Fig. 6. Specific enzymatic activity of wild-type and mutant DPPIV. The ordinate represents the relative DPPIV specific activity calculated from the same protein amounts of wild-type and mutant DPPIV. The amount of protein expressed was determined by scanning the immunoblot loaded with identical amounts of total solubilised protein (blot not show). The activity of wild-type DPPIV was set at 100% and all other values were expressed relative to it. Each column represents the average from four separate experiments. A control experiment of the immunoblot was always run in parallel.

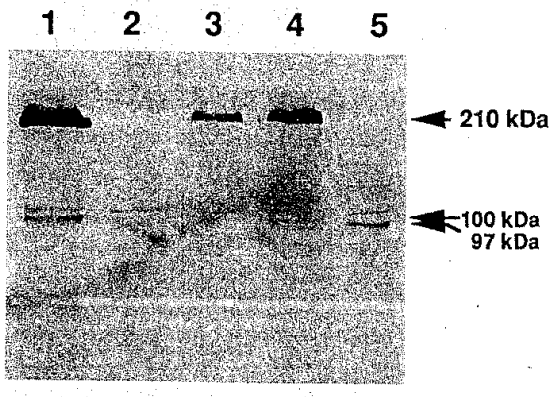

Fig. 7. Analysis of dimerisation of wild-type and mutant DPPIV by non-denaturating SDS/PAGE. The cell solubilizates were incubated with non-denaturating sample buffer containing 60 mM Tris/HCl pH 6.8, 0.003% bromophenol blue, 10% (by vol.) glycerol and 0.5% (mass/vol.) SDS, and not boiled. After SDS/PAGE (7.5%), immunostaining was performed using mAb 13.4. In contrast to CHO cells (lane 2), the wild-type DPPIV (lane 1) shows a major band with molecular mass of about 210 kDa and a minor band at about 100 kDa. Most of [Gln83]DPPIV (lane 4) and of [Gln686]DPPIV (lane 3) migrates to a position corresponding to approximately 210 kDa, whereas [Gln319]DPPIV (lane 5) corresponds to about 97 kDa.

Dimerization of the N-glycosylation mutants of DPPIV. Analysis of wild-type DPPIV and its N-glycosylation mutants by SDS/PAGE under non-denaturating conditions (Fig. 7) revealed that most proteins of [Gln83]DPPIV (lane 4) and [Gln686]DPPIV (lane 3) migrated at a position corresponding to 200–210 kDa, similar to those of wild-type DPPIV (lane 1). Only a small portion of wild-type DPPIV (lane 1) and [Gln83]DPPIV (lane 4) migrated faster, corresponding to about 100 kDa and 97 kDa, respectively. Under the same conditions, however, all of [Gln319]DPPIV remained at a position corresponding to about 97 kDa (lane 5). By cross-linking, it has already been demonstrated that wild-type DPPIV exists as a homodimer of 210 kDa (Jascur et al., 1991). Our results suggest that [Gln83]DPPIV and [Gln686]DPPIV exist mainly as dimers, whereas [Gln319]DPPIV remains monomeric.

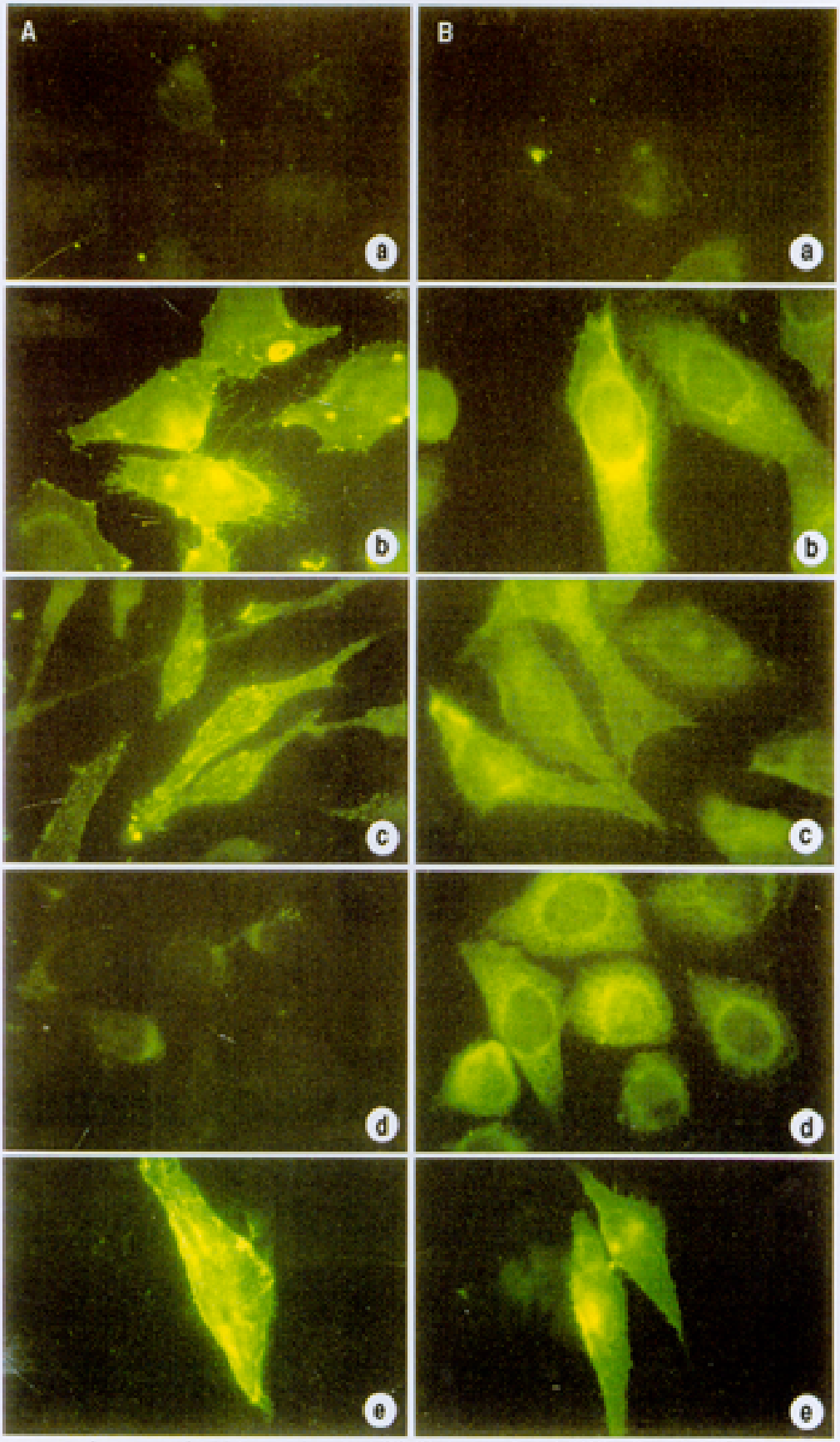

Fig. 8. Immunofluorescence microscopy of wild-type and mutant DPPIV in transfected CHO cells. (A) Cell-surface staining. Cells were fixed with 3% (by vol.) formaldehyde in NaCl/P, at room temperature for 10 min and blocked with 0.1 M glycine in NaCl/P, for 30 min. The anti-DPPIV mAb 13.4 was used for immunostaining at room temperature for 2 h. Visualization was performed with FITC-conjugated rabbit anti-mouse IgG (diluted 1:200) incubated at room temperature for 1 h. While control CHO cells show a negative reaction with DPPIV antibody (a), the CHO/wild-type DPPIV (b), CHO/[Gln83]DPPIV (c) and CHO/[Gln686]DPPIV (e) show surface staining with DPPIV antibody, whereas [Gln319]DPPIV (d) cannot be detected at the cellular surface. (B) Cell permeability staining. Cells were permeabilised with 0.1% Triton X-100 in NaCl/P, at room temperature for 5 min after fixation and before blocking. Immunostaining and detection were performed as above. CHO cells (a) show negative reaction with the DPPIV antibody, whereas CHO/wild-type DPPIV (b), CHO/[Gln83]DPPIV (c), CHO/[Gln319]DPPIV (d), CHO/[Gln686]DPPIV (e) are all positive in this immunoreaction.

Table 1. Comparison of the biochemical properties and functions of wild-type and mutant DPPIV.

DPPIV	Enzyme activity	Cell surface translocation	Half life	N-glycosylation processing	Endo H resistance after 1-h chase	Dimerisation
			h		%	
Wild-type	+	+	50	+	92	+
[Gln83]DPPIV	+	+	25	+	55	+
[Gln319]DPPIV	−	−	5	−	−	−
[Gln686]DPPIV	+	+	35	+	77	+

Intracellular localization of N-glycosylation mutants of DPPIV. Wild-type DPPIV exists as a cell-surface glycoprotein in various cell types in different organisms. The expression of wild-type and mutants DPPIV in CHO cells was investigated using anti-DPPIV mAb 13.4 and polyclonal anti-DPPIV IgG in immunofluorescence microscopy. We have confirmed that non-transfected CHO cells (Fig. 8A, a) are negative in this procedure, whereas wild-type DPPIV (Fig. 8A, b) is expressed on the surface of transfected CHO cells. [Gln83]DPPIV (Fig. 8A, c) and [Gln686]DPPIV (Fig. 8A, e) were also found at the surface of CHO cells. However, the inactive mutant [Gln319]-DPPIV does not appear on the cell surface (Fig. 8A, d). It is located inside the cell, concentrated near the endoplasmatic reticulum (ER) (Fig. 8B, d).

DISCUSSION

Although a large number of studies have been performed on a variety of glycoproteins, using glycosylation inhibitors (Elbein, 1991; Loch et al., 1992), the presence of nonspecific and indirect effects may interfere in the interpretation of the data. In order to investigate the function of the N-glycosylation of a single protein such as DPPIV more specifically, site-directed mutagenesis was used in the present study to abolish the addition of single oligosaccharides to different domains of DPPIV. Three out of eight potential N-glycosylation sites (Asn83, Asn319 and Asn686) were individually replaced by a Gln residue (Fig. 1). Analysis of such DPPIV N-glycosylation mutants was performed in CHO stable transfectants. We found that these serial mutations exert very different effects on the physiological properties and functions of the protein. Each single defective N-glycosylation site of DPPIV resulted in various changes on the enzymatic activity, subcellular localisation and biological stability of the protein in comparison with wild-type DPPIV, as listed in Table 1.

All three mutations affected the biological stability of the protein. The absence of only one of the N-glycosylations was sufficient to reduce the stability of the DPPIV protein. While wild-type DPPIV was relatively stable with an average half-life of about 50 h, all three mutants investigated in the present study exhibited a decreased half-life, although to different degrees (Fig. 5). This result suggests that complete N-glycosylation is important for the protein stability of DPPIV.

It has been demonstrated that N-glycosylation site mutations have a direct impact on the protein stability of several glycoproteins, e.g. mannose-6-phosphate receptor (Wendland et al., 1991), the transferrin receptor (Yang et al., 1993) and acetylcholinesterase (Velan et al., 1993). It is possible that the oligosaccharide side chains protect the protein against protease degradation, as in the case of interferon-γ in which the glycans at Asn25 are critical for protease resistance (Sareneva et al., 1995). Another possible explanation for the decreased stability of the mutants is that the mutations affect the folding and oligomerization process, resulting in a prolonged stay in the ER and an increased proteolytic breakdown in this compartment. The analysis of processing kinetics supports this hypothesis. Although the oligosaccharide side chains of mutants [Gln83]DPPIV and [Gln686]-DPPIV could be processed to a mature complex form, the processing of both mutants was significantly slower than that of wild-type DPPIV (Fig. 4).

The Asn319 mutation of DPPIV has a marked influence on protein stability and enzyme activity, and severely affects protein transport. Several experiments have suggested that N-linked carbohydrates are required for stability of the enzyme structure (Haraguchi et al., 1995) and correct intracellular trafficking (Gieselmann et al., 1992; Olivares et al., 1995; Garcia Rodriguez et al., 1995). In SDS/PAGE the [Gln319]DPPIV appeared as a band of lower molecular mass (Fig. 3), and it was converted to a polypeptide of 84 kDa after endo H digestion (Fig. 4). Therefore this molecule presumably contains only high-mannose-type N-glycans, and these are not processed into mature complex oligosaccharides. Furthermore, [Gln319]DPPIV is unable to dimerize (Fig. 7). In general, plasma membrane glycoproteins tend to be oligomers, and it is assumed that oligomerization occurs in the ER. It has been postulated that oligomerization of membrane proteins is essential for export from the ER (Copeland et al., 1988; Kreis and Lodish, 1986; Hurtley and Helenius, 1989). Therefore, the inability of [Gln319]DPPIV to dimerize may be the reason for its retention in the ER.

Recently it has been suggested that dimerization of wild-type DPPIV takes place in the late-Golgi compartment (Jascur et al., 1991). This is in conflict with the observation that most proteins are oligomerized in the ER. Some investigators have suggested that glycosylation of glycoproteins occurs in the ER because oligosaccharides are required to facilitate efficient folding and quality control of newly synthesized proteins (Kornfeld and Kornfeld, 1985; Danielsen, 1992; Marquardt and Helenius, 1992; Helenius, 1994). Only correctly folded and assembled proteins would then be selectively transported from the ER to the Golgi compartment and beyond. This perspective is supported by our experiments, as well as by other recent work (Letourneur et al., 1995). In the sequence of wild-type DPPIV, Asn319 is located within a cysteine-rich region. For most glycoproteins, the folding process involves the formation of disulfide bridges. It may be assumed that the prevention of N-glycosylation at position Asn319 leads to a misfolding, for example, by formation of aberrant interchain disulfide bonds (Marquardt and Helenius, 1992; Helenius, 1994). The resulting abnormal conformation is recognized as a signal for the retention and degradation in the ER (Klausner and Sitia, 1990; Sousa and Parodi, 1995). This hypothesis was supported by the results of our pulse/chase experiments, showing that the mutant [Gln319]DPPIV was quickly degraded after polypeptide synthesis (Fig. 5). Thus, incorrect folding and retention in the ER may be the reason for the defective dimerization of this mutant protein.

Both [Gln83]DPPIV and [Gln686]DPPIV expressed enzymatic activity (Fig. 6), despite the fact that Asn686 is located

close to the active site (Ogata et al., 1992; David et al., 1993). In contrast, [Gln319]DPPIV showed hardly any enzymatic activity (Fig. 6). This suggests that the conformation of the active site of mutants [Gln83]DPPIV and [Gln686]DPPIV are not influenced, whereas the conformation of [Gln319]DPPIV may be dramatically changed by its incorrect folding.

In conclusion, our results show that single defective N-glycosylations in different domains of DPPIV have widely different effects on the subcellular localisation and biochemical properties of the protein. Each of the three N-glycans of DPPIV studied contributes to biological stability and processing. This suggests that a cotranslational N-glycosylation of wild-type DPPIV is important for the attainment of a correctly folded, stable conformation. However, the degree of dependence is variable. Deletion of the Asn319 oligosaccharide chain of DPPIV resulted in the retention of an enzymatically inactive monomeric protein within the ER, and this protein was rapidly degraded. Since Asn319 is located in a cysteine-rich domain, it is proposed that this mutant protein may be misfolded by the formation of aberrant intrachain disulfide bonds.

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