Structural Requirements for Catalysis, Expression, and Dimerization in the CD26/DPIV Gene Family

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ABSTRACT: Dipeptidyl peptidase IV (DP-IV/CD26), fibroblast activation protein (FAP), DP-like 1 (DPL1), DP8, DP9, and DPL2 comprise the CD26 gene family. CD26/DP-IV has roles in liver disease, T cell costimulation, chemokine biology, type II diabetes, and tumor biology. DPIV substrates include the glucagonlike peptides, neuropeptide Y, and the chemokines CCL3, CCL5, CCL11, CCL22, and CXCL12. We have proposed that the extracellular region of CD26 is analogous to prolyl oligopeptidase in consisting of an α/β hydrolase domain contributed by both N- and C-terminal portions of the polypeptide and a seven-blade β-propeller domain. Replacing the C-terminal portion of the predicted α/β hydrolase domain of CD26 (residues 501−766) with the homologous portion of DP8 or DP9 produced intact proteins. However, these chimeric proteins lacked dimerization and peptidase activity, suggesting that CD26 dimerization requires the C-terminal portion of the α/β hydrolase domain. Deleting some N-terminal residues of the α/β hydrolase domain of CD26 ablated peptidase activity and greatly diminished cell surface expression. Together with previous data that CD26 peptidase activity requires the C-terminal 20 residues, this suggests that peptidase activity requires the entire α/β hydrolase domain. The catalytic triad of DP8 was shown to be Ser^{739}−Asp^{817}−His^{849}. Glu^{259} of DP8, a residue distant from the catalytic triad yet greatly conserved in the CD26 gene family, was shown to be required for peptidase activity. These data concord with our predicted CD26 structure, indicate that biosynthesis of a functional fragment of CD26 is difficult, and confirm the functional homology of DP8 with CD26.

CD26 is a ubiquitous, multifunctional type II cell surface glycoprotein also present in serum and semen as a soluble form. CD26 has a post-proline dipeptidyl aminopeptidase (DP)[1]activity (DPIV; E.C. 3.4.14.5) that has become interesting in the fields of immunology, endocrinology, cancer biology, and nutrition. The natural substrates of DPIV/CD26 include the chemokines CCL3, CCL5, CCL11, CCL22, and CXCL12; the glucagonlike peptides; and neuropeptide Y. The biology of CD26 has been reviewed recently (*1*−*5*). CD26 mediated cleavage of chemokines reduces signaling via Th2 chemokine receptors more than Th1 and monocyte chemokine receptors, whereas the overall impact of CD26 derived peptidase activity on inhibition of HIV infection by chemokines is unclear (*3*, *6*, *7*). For example, CD26 is coexpressed with CXCR4 on human blood derived T cells (*8*) and inactivates the CXCR4 ligand CXCL12 (*9*, *10*). Inhibitors of the DPIV enzyme activity of CD26 have potential as a novel therapy for type II diabetes (*4*, *11*, *12*).

CD26, DP8, DP9, fibroblast activation protein (FAP), DP-like1 (DPL1; DP6; DPX), and DPL2 form the CD26 gene family, and those with DP activity (CD26, DP8, DP9, and FAP) form the S9b peptidase family (*13*−*15*). S9b members exhibit 27−60% amino acid identity with each other. The three-dimensional structure of the related S9a peptidase prolyl oligopeptidase (POP, E.C. 3.4.21.26) has been solved (*16*) and has been used as a template for predicting CD26 structure (*3*, *13*). We have predicted that the extracellular 738 residues of CD26, a 766-residue protein, form an α/β hydrolase fold consisting of residues 29−132 and 502−766 and a seven-blade β-propeller fold consisting of residues 133−501 (*3*). The propeller covers the catalytic cleft of the hydrolase domain and in POP is a gating filter of substrates (*17*). We have shown that Glu^{205} and Glu^{206} of CD26 are essential for peptidase activity and postulated that these glutamates have a role in substrate entry (*18*). Glu^{205} is within a conserved motif (Figure 1) and is conserved in all enzymes of the CD26 gene family (*15*). The catalytic triad has been confirmed by point mutation of mouse CD26 as Ser^{624}, Asp^{702}, and His^{734} (*19*), equivalent to Ser^{630}, Asp^{708}, and His^{740} of human CD26. The ancestral gene of the CD26 family was probably most closely related to DP8 (*15*), so we tested whether these residues are essential for catalysis in DP8.

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[1] Abbreviations: DP, dipeptidyl peptidase; FAP, fibroblast activation protein; POP, prolyl oligopeptidase; ADA, adenosine deaminase.

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CD26	201	W	V	Y	E	E	E
DP8	254	F	V	L	Q	E	E
DP9	244	F	V	I	Q	E	E
FAP	199	W	V	Y	E	E	E
DPL1	290	W	L	Y	E	E	E
DPL2	222	W	L	Y	E	E	E

FIGURE 1: Conserved motif containing a conserved pair of glutamic acids in the propeller domain of the CD26 family.

In the absence of a crystal structure of CD26, some understanding of its structure −function relationships has been made using point mutations and truncations of CD26 (*13*, *18*, *20*−*22*), rat-human chimeric CD26 proteins (*22*), a model of CD26 (*3*, *13*), and characterization of the related molecules FAP (*23*, *24*), DP8 (*25*), and DP9 (*26*). Truncation of only 20 residues from the CD26 C-terminus ablates enzyme activity (*13*), but N-terminal truncations of up to 34 residues retain both enzyme and ADA binding activities (*13*, *27*−*29*). The N-terminal portion of the α/*β* hydrolase domain of POP has only 15% identity with CD26, and a model of the structure of this portion of CD26 has not been made (*3*). However, it is reasonable to predict by analogy with POP that the residues of CD26 between the *β*-propeller and the transmembrane domains form part of the α/*β* hydrolase fold (Figure 2). We reasoned that deletion of this portion of CD26 would either show it to be essential for peptidase activity or suggest the possibility of a truncated form of CD26 that is active. The existence of such a form would indicate a potential to solve the structure of a CD26 fragment.

Carbohydrate moieties can reduce the propensity of a glycoprotein crystal to yield useful diffraction data, and CD26 has nine potential N-linked glycosylation sites and is about 25% carbohydrate of variable composition (*30*). Therefore, a further purpose in deleting residues and in making chimeras with the CD26 relatives that lack (DP8) or have little (DP9) glycosylation was to remove some glycosylation sites, at positions 85, 92, 150, 520, and 685.

Here we report novel mutational data pertinent to understanding structure−function relationships in proteins of the CD26 family. The predicted catalytic triad of DP8 (*25*) was tested by single amino acid point mutation. In addition, a conserved residue in the predicted *β*-propeller of DP8, Glu^{259}, was shown to be essential for catalytic activity. Both N- and C-terminal portions of the α/*β* hydrolase domain of CD26, residues 36−103 and 502−766, were shown to be essential for catalytic activity and intact structure. Furthermore, the C-terminal portion of the CD26 hydrolase domain, residues 502−766, was found to be essential for dimerization.

EXPERIMENTAL PROCEDURES

Materials. The wild-type cDNAs used were CD26 (*33*), DP8 (*25*), and DP9 (*26*), which have GenBank accession numbers M80536, AF221634, and AF542510, respectively. Plasmid DNA was extracted using the Rapid Pure Miniprep Kit (Bio101, Vista, CA), the QIAGEN Midiprep Kit (QIAGEN, Hilden, Germany), or the JETSTAR Plasmid Maxiprep Kit (GENOMED, Germany). The QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce point mutations in the plasmid DNA. Constructs were sequence verified using automated sequencing with fluorescent dideoxy terminators (SUPAMAC, Sydney, Australia). Restriction enzymes were purchased from Roche (Palo Alto, CA) or MBI Fermentas (Hanover, MD). Oligonucleotides were purchased from Sigma (Sydney, Australia). The monoclonal antibodies (mAb) to CD26 TA5.9 (*31*), 2A6 (*32*) and B10/EF6 (*30*), and the antiserum to ADA (*18*) have been described. Electrophoresis gels and buffers were purchased from Novex (San Diego, CA) and Gradipore (Sydney, Australia). Immobilon-P PVDF membranes were purchased from Millipore (Bedford, MA). Visualization of immune complexes following Western blotting used chemiluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and BIOMAX ML film (Kodak, Rochester, NY).

Preparation of CD26 Deletion Mutants. A ClaI restriction site (ATC GAT) was introduced starting at position 110 (after amino acid 35) of the wild-type CD26 sequence using mutagenic oligonucleotides ClaI_site_Forward (CAA AGG CAC AGA TAT CGA TAC AGC TGA CAG TCG) and ClaI_site_Reverse (CGA CTG TCA GCT GTA TCG ATA TCT GTG CCT TTG). The point mutation was performed in the wild-type CD26 construct in pcDNA3.1 (Invitrogen, Carlsbad, CA). This construct was named pcDNA3.1/ClaI/CD26. CD26 deletion mutants were prepared by polymerase chain reaction (PCR) using primers designed to introduce a ClaI restriction site and an ApaI restriction site at the 5′ and 3′ ends, respectively, of each excision (Table 1). The natural stop codon of CD26 was retained along with 600 base pairs of 3′ noncoding sequence. The ClaI/ApaI deletion fragments were then cloned into the ClaI/ApaI digest of pcDNA3.1/ClaI/CD26.

Preparation of Single Amino Acid Point Mutations in DP8. Point mutations were performed in the previously described (*25*) wild-type DP8 pcDNA3.1/DP8-V5-His construct to introduce single amino acid changes in DP8 using mutagenic oligonucleotides (Table 1). Fusion constructs were created such that the V5 epitope was expressed and available for detecting recombinant protein.

Construction of CD26-DP8 and CD26-DP9 Chimeras. Point mutations were performed in the wild-type CD26 construct in pcDNA3.1 (*25*) to introduce a ClaI restriction site (ATC GAT) starting at position 2482 (after amino acid 501) and an XbaI restriction site (TCT AGA) 5′ of the start of the CD26 coding sequence. These steps used the mutagenic oligonucleotides CD26ClaI_site_Forward (CCT GGA AGA CAA TTC AGC TAT CGA TAA AAT GC), CD26ClaI_site_Reverse (GCA TTT TAT CGA TAG CTG AAT TGT CTT CCA GG), XbaI_site_KO_Forward (CCG CTC GAG TCT GGA CGC CGA CGA TG), and XbaI_site_KO_Reverse (CAT CGT CGG CGT CCA GAC TCG AGC GG). These steps made ClaI and XbaI unique restriction sites for constructing chimeric molecules. DP8 and DP9 DNA inserts were prepared by PCR using primers designed to introduce a ClaI restriction site and an XbaI restriction site at the 5′ and 3′ ends, respectively (Table 1). The natural stop codons of DP8 and DP9 were retained.

Cell Culture and Transfection. Cultivation and transfection of monkey kidney fibroblast lines COS-6 (CRL-1654, ATCC), COS-7 (CRL-1651, ATCC), and the human kidney epithelial line, HEK293T (ATCC, CRL-11268) were carried out as described previously (*18*). Three or more clones of each expression construct were transfected into cells, and the clone that produced the greatest proportion of expressing cells was used for further study. Stable COS-7 cell lines

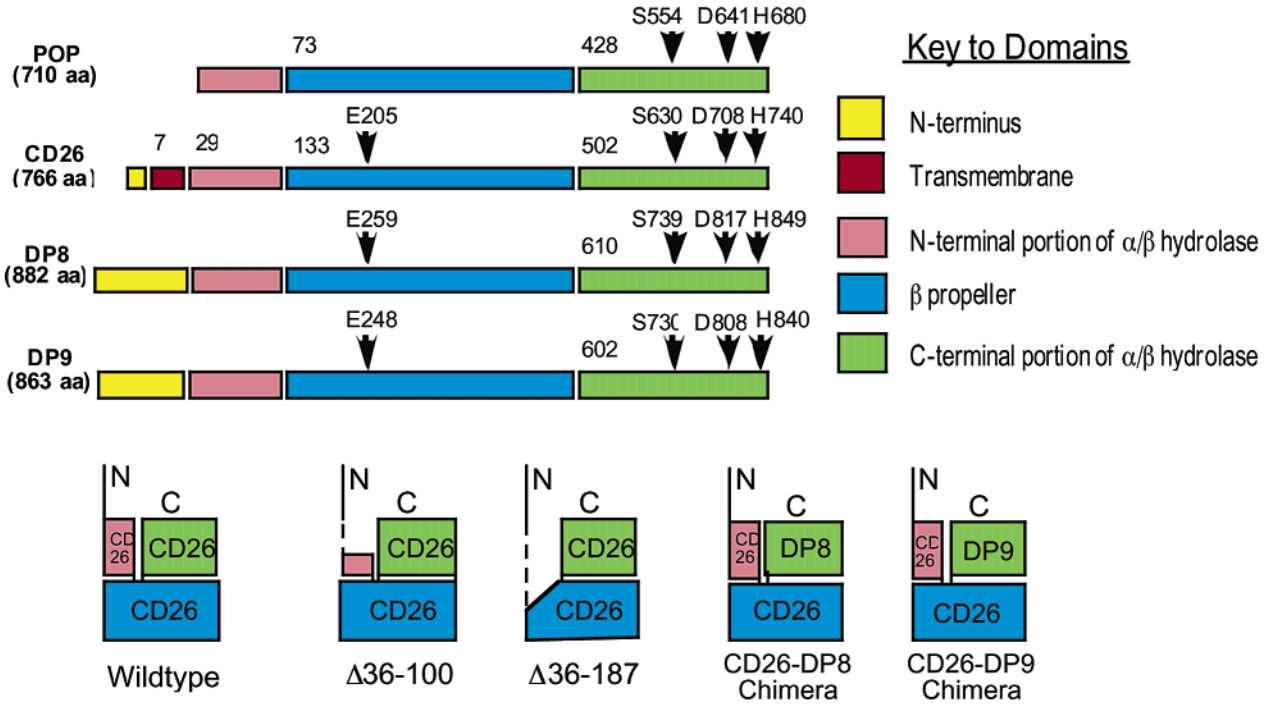

FIGURE 2: Schematic of POP, CD26, DP8, and DP9 proteins and of CD26 mutants. The arrangement of domains and some residues of interest are depicted. The CD26 deletion mutants retained the N-terminal cytoplasmic and transmembrane domains. Diagrams depict the smallest (Δ36−100) and largest (Δ36−187) deletions from CD26, with the deleted section indicated by a dashed line. The chimeric proteins were created by replacing the C-terminal portion of the α/β hydrolase domain of the CD26 molecule with that of DP8 or DP9. Not to scale.

Table 1: Primer Sequences

	primer sequence (5′ ⟹ 3′)
	Forward Primers
CD26_ClaI_Δ36−100	GGC CGG ATC GAT TCT ATC AAT GAT TAT TC
CD26_ClaI_Δ36−103	GGC CGG ATC GAT GAT TAT TCA ATA TCT CCT G
CD26_ClaI_Δ36−157	GGC CGG ATC GAT TCA CCA GTG GGT CAT AAA TTG GC
CD26_ClaI_Δ36−161	GGC CGG ATC GAT CAT AAA TTG GCA TAT GTT TG
CD26_ClaI_Δ36−180	GGC CGG ATC GAT GCT TCA TAT GAC ATT TAT G
CD26_ClaI_Δ36−187	GGC CGG ATC GAT CCA AGT TAC AGA ATC ACA TGG
DP8E259K_forward	CCT TTG TTC TCC AAA AGG AAT TTG ATA G
DP8S739A_forward	CCA CGG CTG GGC CTA TGG AGG ATA C
DP8D817A_forward	CAT GGT TTC CTG GCT GAG AAT GTC C
DP8H849A_forward	CCT CAG GAG AGA GCC AGC ATA AGA G
DP8_ClaI_Forward	GGG CGG ATC GAT TCA GCA GGT CCT CTT C
DP9_ClaI_Forward	CGC CCC ATC GAT GCA GCC AGC TG
	Reverse Primers
CD26_ApaI_Reverse	GCC GCG ATC CTA GAG GGC CCT TCG AA
DP8E259K_reverse	CTA TCA AAT TCC TTT TGG AGA ACA AAG G
DP8S739A_reverse	GTA TCC TCC ATA GGC CCA GCC GTG G
DP8D817A_reverse	GGA CAT TCT CAG CCA GGA AAC CAT G
DP8H849A_reverse	CTC TTA TGC TGG CTC TCT CCT GAG G
DP8_XbaI_Reverse	GCG GCC TCT AGA TTA TAT CAC TTT TAG AGC AG
DP9_XbaI_Reverse	GGC GCG TCT AGA GGG CGG GAC AAA GTG CCT CAC TGG

expressing DP8 wild-type and DP8 mutants were selected by adding 50 μg/mL Genticin (G418) to the medium from 24 h after transfection.

Detection of Protein Expression and ADA Binding. Cell suspensions were stained with anti-CD26 mAb followed by phycoerythrin (PE) conjugated goat anti-mouse immunoglobulin (Caltag, Burlingame, CA) for flow cytometry as described previously (*18*). Immunoperoxidase cytochemistry with antibodies and biotinylated ADA on ethanol-fixed cells has been described previously (*18*). Immunofluorescence cytochemistry used Alexa 594 conjugated rabbit anti-mouse immunoglobulin (Molecular Probes, Eugene, OR) in conjunction with Wheat Germ Agglutinin-FITC (Molecular Probes), diluted 1:100, and DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) stained. The fluorescent staining was visualized using an AxioSkop fluorescent microscope attached to an AxioCam digital camera (Carl Zeiss, Munich, Germany). The digital images were manipulated using AxioVision version 3.0 (Carl Zeiss) and Canvas version 7 (Deneba, Miami, FL). Western blotting was essentially as described previously (*18*) except that incubation was in sample buffer at either 40 or 60 °C, and prestained broad range SDS−PAGE standards catalog number 161-0318 (Biorad, Hercules, CA) and horseradish peroxidase conjugated anti-rabbit Ig catalog number P0260 (Dako, Santa Barbara, CA) were used.

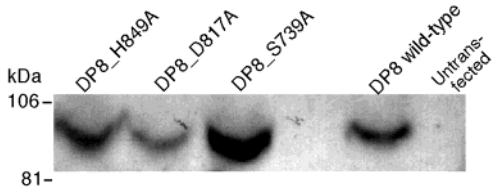

FIGURE 3: Western blot of 8% SDS−PAGE of stable cell lines expressing wild-type or single amino acid point mutations of DP8. Soluble extracts of 1.25×10^6 COS-7 cells were loaded into each lane. All mutants showed the same mobility as wild-type DP8, detecting a 100 kDa protein using a mAb to the V5 epitope tag on the C-terminus.

Enzyme Assays. DPIV enzyme activity was measured using a 1-h incubation at 37 °C and 1 mM Gly-Pro-nitroanilide-HCl (Sigma) in 0.1 M Na_2HPO_4/NaH_2PO_4 (phosphate buffer) (pH 6.7), 1 mM Ala-Pro-*p*-nitroanilide (Ala-Pro-*p*NA, Bachem AG, Bubendorf, Switzerland), and 1mM Ala-Pro-AFC (Bachem) in 0.1 M phosphate buffer (pH 7.4). For the chromogenic substrates, absorbance was measured in dual wavelength mode at 405 and 690 nm using a Titertek Multiskan MCC/340 (EFLAB, Finland). Fluorescence was measured with 405 nm excitation and 510 nm emission using a Victor2 1420 multilabel counter (Wallac, Turku, Finland). Raw measurements from control-transfected cells were subtracted prior to kinetics calculations (KaleideGraphTM v. 3.5.1, Synergy, Reading, PA). Monolayers of COS cells and cytocentrifuged 293T cells were enzyme stained using Fast Blue RR (4-benzoyl-amino-2,5-dimethoxy aniline-$ZnCl_2$; Sigma) in 0.04 M phosphate buffer mixed with Gly-Pro-4-methoxy-β-naphthylamide-HCl (Sigma) in *N′N′*-dimethyl formamide (DMF) or Ala-Pro-4-methoxy-β-naphthylamide-HCl (Sigma) in methanol. Prior to cytoplasmic staining, cells were permeabilised by incubation in 0.1% Tween 20 (Sigma) in PBS for 10 min at ambient temperature.

RESULTS

Four Residues Essential for DP8 Peptidase Activity. The 882-residue polypeptide DP8 has 27% identity with CD26, and the ancestral gene of the CD26 family was probably DP8-like (*15*). Therefore, a sequence alignment was used to predict residues of DP8 essential for DP8 catalytic activity (*25*). Testing these predictions tested both the assertion that DP8 is a peptidase homologous to CD26/DPIV and our prediction of the tertiary structure of CD26. We previously predicted that Ser^{739}, Asp^{817}, and His^{849} would form the catalytic triad in DP8 and that Glu^{259} is an additional requirement for DP8 peptidase activity (*25*). Glu^{259} of DP8 aligns with Glu^{205} of CD26. Glu^{205} of CD26 is essential for peptidase activity (*18*), but the mechanism of its contribution to this activity is unknown.

The dependence of DP8 peptidase activity upon all four of these residues was shown by cell stain and Ala-Pro-AFC enzyme assays of transient and stable transfectants. On comparable numbers of successfully transfected COS7 cells, the sensitive Ala-Pro-AFC assay generated a wild-type K_m of 0.13 ± 0.03 mM and a V_{max} of 143 ± 7.2 ΔA_{405} min^{-1} versus a K_m of 1.7 ± 0.6 mM and a V_{max} of 11 ± 1.5 ΔA_{405} min^{-1} from the Glu-259-Lys mutation ($n = 4$) and no detectable activity from the catalytic triad mutations. The wild-type and mutant DP8 molecules were of intact size (Figure 3) and cytoplasmic location (immunocytochemistry not shown). Consideration of the enzyme cytochemistry data and immunoblots, flow cytometry, and immunocytochemistry with an antibody to the V5 epitope tag indicated that these recombinant proteins retained the C-terminal epitope tag. All the stable transfectants produced little DP8 (Figure 3) because fewer than 2% of cells, including transfectants of wild-type CD26 and DP8, stably expressed recombinant protein. Therefore, further experiments used transient transfection.

Table 2: Deletion Mutants of CD26

name of mutation	enzyme activity[b]	MAb binding[a] EF6/B10	2A6	TA5.9	ADA binding[c]
CD26 wild-type	+++[d]	+++	+++	+++	+++
CD26_ClaI_Δ36−100	none	none	+	++	+
CD26_ClaI_Δ36−103	none	none	+	++	+
CD26_ClaI_Δ36−157	none	none	none	none	none
CD26_ClaI_Δ36−161	none	none	none	none	none
CD26_ClaI_Δ36−180	none	none	none	none	none
CD26_ClaI_Δ36−187	none	none	none	none	none

[a] Flow cytometry and immunocytochemistry. [b] Enzyme cytochemistry. [c] Flow cytometry with biotinylated ADA. [d] Staining intensity indicated by + ($n = 3-5$).

CD26 Deletion Mutations. A common approach to solving the tertiary structure of a large protein is to express an individual domain, so an aim of studying deletion mutants was to investigate the potential for expressing a structurally intact fragment of CD26. Intact soluble forms of CD26, which lack the cytoplasmic and transmembrane domains up to residue 34, have been expressed and found to retain the antibody binding, ADA binding, and catalytic and dimerization activities of wild-type CD26 (*28*, *29*, *32*, *34*, *35*). In contrast, in the present experiments deletions were made in extracellular regions of CD26 (Figure 2). The N-terminal 35 residues, which comprise the cytoplasmic and transmembrane domains and are sufficient to permit normal cytoplasmic trafficking and cell surface expression of these mutant proteins, were retained so that cell surface expression became an additional test of protein integrity. The propeller blade is a repeated unit (*36*). Accordingly, locations of deletion mutations were chosen using internal sequence alignments so that complete rather than partial predicted blades could be deleted. Two mutants were made that had deletions of portions of the α/β hydrolase domain (Δ36−100, Δ36−103), while the other four mutants additionally had small portions of the predicted β-propeller domain deleted (Table 2, Figure 2).

The two mutants that lacked N-terminal portions of the α/β hydrolase domain were poorly cell surface expressed, reflected in their limited ability to bind mAb 2A6, mAb TA5.9, or ADA (Figures 4 and 5, Table 2). MAb EF6/B10 recognizes a conformationally dependent epitope formed by CD26 residues 117−187 (*21*), so the inabilities of the deletion mutants to either bind mAb B10 or exhibit detectable peptidase activity indicated disruption of the structure in this region. Deletions of residues 36−157 or 36−161, predicted to delete a small portion of the predicted β-propeller domain in addition to deletion of the N-terminal portion of the α/β hydrolase domain (Δ36−157 or Δ36−161), coincided with loss of both ADA binding and antibody binding (Figures 4 and 5, Table 2), indicating that the region 104−156, which includes a glycosylation site at 150, is essential for biosynthesis of the CD26 molecule. The data presented is representative of three replicate transfection experiments.

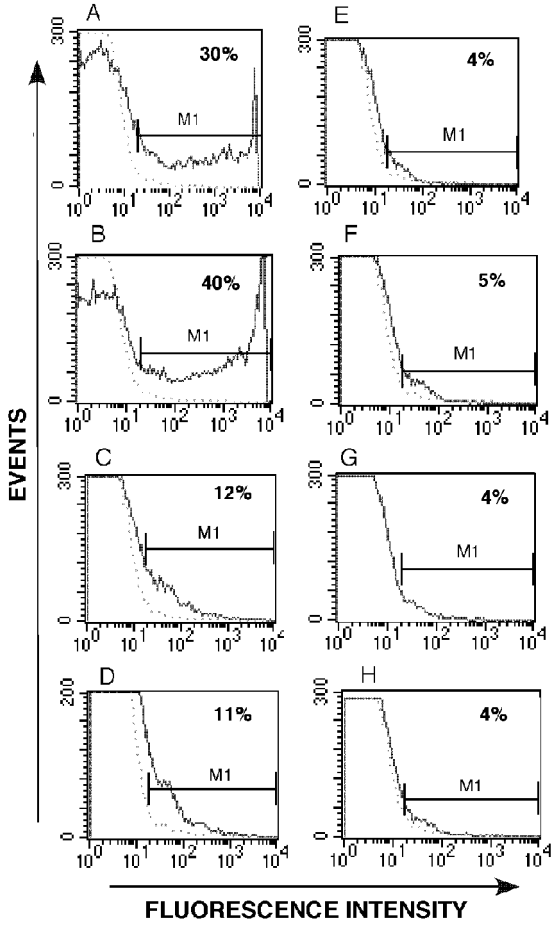

FIGURE 4: Cell surface expression of CD26 deletion mutants. COS-6 cells transfected with wild-type CD26 (A), wild-type CD26 with ClaI mutation (B), or a deletion mutant (C−H) were cell surface immunostained using anti-CD26 mAb TA5.9 then analyzed by flow cytometry. The deletion mutants CD26_ClaI_Δ36−100 (C) and CD26_ClaI_Δ36−103 (D) bound anti-CD26 mAb TA5.9. In contrast, deletion mutants CD26_ClaI_Δ36−157 (E), CD26_ClaI_Δ36−161 (F), CD26_ClaI_Δ36−180 (G), and CD26_ClaI_Δ36−187 (H) did not specifically bind mAb TA5.9. The negative control of untransfected cell data is a dotted line. The percentages of immunopositive cells are shown.

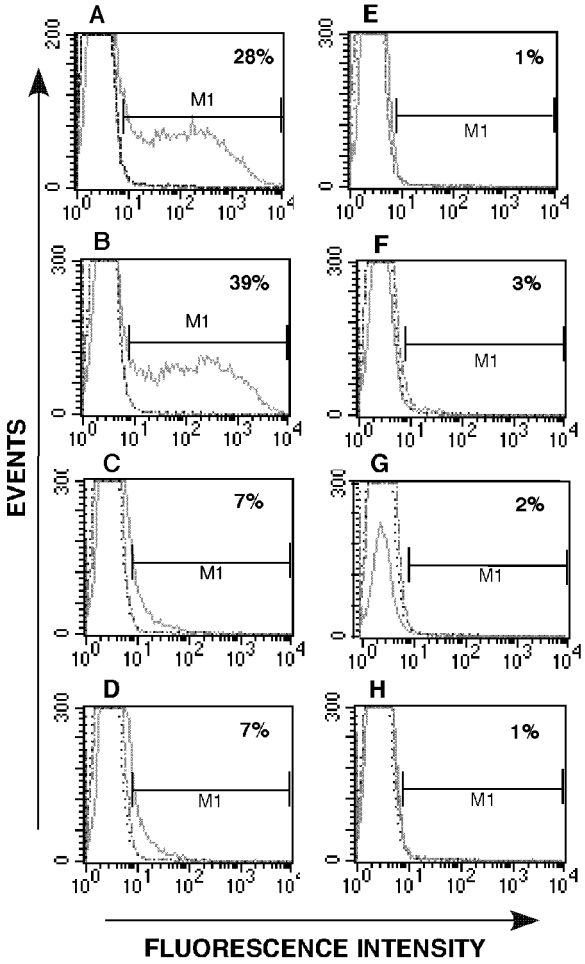

FIGURE 5: ADA binding to CD26 deletion mutants. COS-6 cells transfected with wild-type (A), wild-type with ClaI mutation (B), or deletion mutant (C−H) were cell surface immunostained using ADA followed by rabbit anti-ADA then analyzed by flow cytometry. The deletion mutants CD26_ClaI_Δ36−100 (C) and CD26_ClaI_Δ36−103 (D) weakly bound ADA. In contrast, deletion mutants CD26_ClaI_Δ36−157 (E), CD26_ClaI_Δ36−161 (F), CD26_ClaI_Δ36−180 (G), and CD26_ClaI_Δ36−187 (H) did not bind ADA. The negative control of untransfected cell data is a dotted line. The percentages of immunopositive cells are shown.

CD26-DP8 and CD26-DP9 Chimeras. The level of structural and functional homology between CD26 and DP8 and DP9 was investigated by making chimeric molecules in which the C-terminal portion of the CD26 α/β hydrolase domain (residues 502−766; see Figure 2) was replaced by the equivalent residues of DP8 or DP9. Data from three replicate cDNA clones of the CD26-DP9 chimera, designated the names 4.1, 4.2, and 10.3, were obtained. The CD26-DP8 and CD26-DP9 chimeric constructs encoded 776 and 767 amino acid proteins, respectively, which exhibited mobilities of about 100 and 105 kDa, respectively, on SDS−PAGE (Figure 6). The chimeras probably ran faster than wild-type CD26 monomers because the chimeras lacked two of the potential N-linked glycosylation sites of CD26, at Asn520 and Asn685. DP8 lacks potential glycosylation sites, and DP9 has just one in the hydrolase domain. Unlike CD26, the chimeras did not dimerize. The CD26 dimer exhibited its usual Mr of about 170 kDa (*13*, *32*, *37*−*40*). The chimeras and CD26 monomer and dimer bound to ADA (Figure 6C).

All chimeric proteins were detectable using anti-CD26 mAbs (Figures 6−8). The mAbs TA5.9 and B10 bind conformationally dependent epitopes formed by CD26 residues 324−552 and 117−187, respectively (*13*, *21*). Therefore, the flow cytometry data (Figure 7) showed that, like CD26, the chimeric proteins were expressed intact and in readily detectable quantities on the surface of transfected cells. These data concorded with the immunocytochemistry and enzyme cytochemistry (Figures 8 and 9). Interestingly, these data showed that the TA5.9 epitope does not contain CD26 residues 502−552, concordant with our proposed location of this epitope on the β-propeller domain (*13*). The chimeric proteins lacked peptidase activity (Figure 9).

DISCUSSION

Structure−function relationships in CD26 have been examined using point mutations and truncations of CD26 (*13*, *18*, *20*−*22*), rat-human chimeric CD26 proteins (*22*), a model of CD26 (*3*, *13*), and characterization of the related molecules FAP (*23*, *24*), DP8 (*25*), and DP9 (*26*). Deletions, chimeras, and mutations were used here to increase the understanding of the CD26 gene family. The predicted

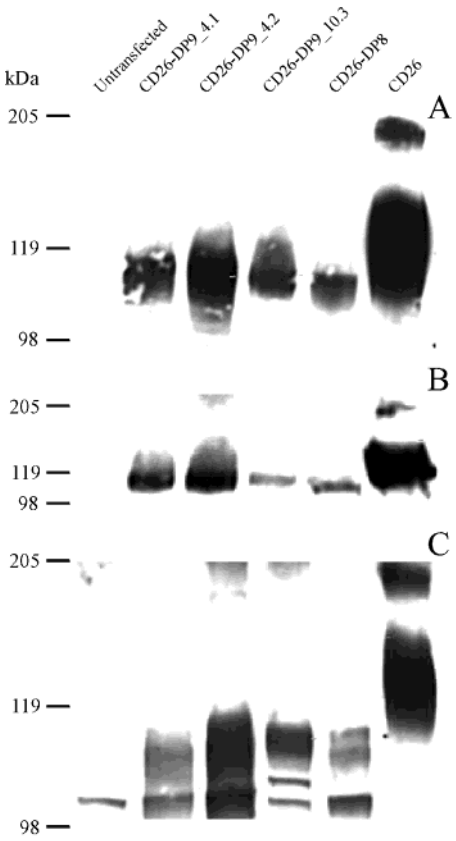

FIGURE 6: CD26-DP8 and CD26-DP9 chimeric proteins did not dimerize. Membrane preparations of 1×10^6 293T cells per lane were incubated in sample buffer at 60 (A and C) or 40 °C (B) then run on 8 (A and C) or 8−16% (B) SDS−PAGE and immunoblotted using anti-CD26 mAb 2A6 (A and B) or ADA and an anti-ADA antiserum (C). CD26-DP9_4.1, CD26-DP9_4.2, and CD26-DP9_10.3 are three replicate DNA clones of the CD26-DP9 chimera. Wild-type CD26 dimer ran at the expected mobility of about 170 kDa. Representative data from six replicate transfections.

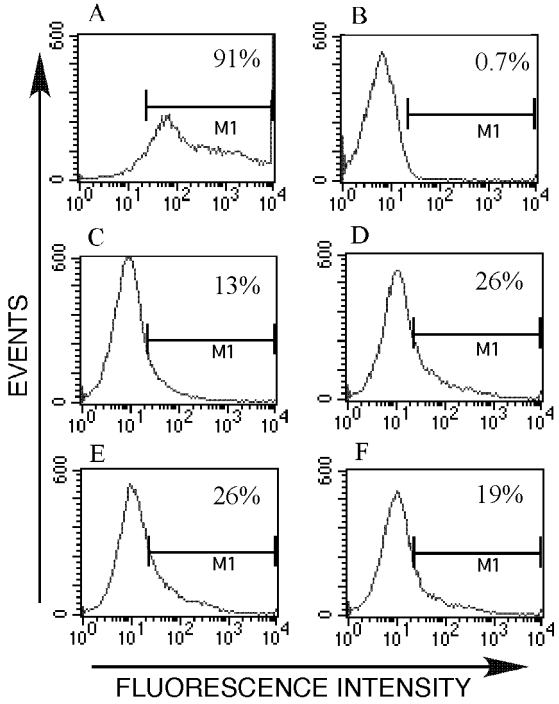

FIGURE 7: Cell surface expression of CD26-DP8 and CD26-DP9 chimeras. Transfected 293T cells were stained with a 1:1 mixture of the anti-CD26 mAbs TA5.9 and EF6/B10 followed by Texas Red conjugated goat anti-mouse Ig. Wild-type CD26 (A), untransfected cells (B), and CD26-DP8 chimera (C); and the three replicate CD26-DP9 chimeras CD26-DP9_4.1 (D), CD26-DP9_4.2 (E), and CD26-DP9_10.3 (F). Representative data from three replicate transfections. The percentage of cells immunopositive is shown in each panel.

catalytic triad of DP8 (*25*) was confirmed. In addition, a conserved glutamic acid residue in the β-propeller domain of DP8, Glu[259], which is greatly conserved in this gene family (Figure 1), was shown to be essential for catalytic activity. The only proteins of the CD26 gene family that have a substitution of this conserved glutamic acid are a subset of those that diverge from the GWSYG motif around the catalytic serine and are thus very unlikely to have peptidase activity (*15*). Both N-and C-terminal portions of the α/β hydrolase domain of CD26, residues 36−103 and 502−766, were shown to be essential for catalytic activity and intact structure. Furthermore, the C-terminal portion of the hydrolase domain, which is residues 502−766, was found to be essential for dimerization.

Our chimera data are the first information pertinent to the dimerization mechanism and suggest that dimerization depends on the C-terminal portion of the hydrolase domain. As the catalytic triad in each chimera was contributed by DP8 or DP9, which are active as monomers, the data also indicate that the N-terminal portion and/or propeller of CD26 are incompatible with DP8 and DP9 activity. CD26 runs on SDS−PAGE as a monomer at 110 kDa Mr and as a dimer at about 170 kDa Mr. The cause of the CD26 dimer exhibiting greater than expected mobility in SDS−PAGE is unknown. The dimer is catalytically active, whereas the monomer is not (*39*, *40*). CD26 and FAP are converted to monomer by heat treatment (reviewed elsewhere, ref *7*). The ratio of CD26 dimer to monomer varies between organs (*37*) and could relate to a mechanism of controlling DPIV activity. Further study of dimerization is warranted.

About 25% of the mass of CD26 is carbohydrate, but the relative contributions of the nine potential N-linked glycosylation sites are not well-understood. The CD26 gene family lacks potential sites for other types of glycosylation. DP8 lacks glycosylation, and DP9 has one potential glycosylation site in the peptidase domain, 211 residues from the C-terminus. Therefore, our observation that the Mr of the CD26 monomer was greater than those of the CD26-DP8 and CD26-DP9 chimeras suggests that the potential glycosylation sites in the C-terminal portion of CD26, Asn520, and Asn685, are glycosylated.

Single amino acid point mutations of three of the eight potentially glycosylated asparagines of rat CD26 have shown that glycosylation of Asn319 but neither Asn83 (human Asn85) nor Asn686 (human Asn685) is required for cell surface expression or peptidase activity (*20*). A crystal structure of CD26 might be more readily determined if the extent of glycosylation could be reduced. However, the C-terminal chimeras and N-terminal deletions reported here reduced the number of glycosylation sites but, like this previous attempt (*20*), produced either inactive or significantly glycosylated CD26.

We propose that the Δ36−101 and Δ36−103 mutants lacked most of the N-terminal portion (residues 29−132) of the α/β hydrolase domain. This portion of the α/β hydrolase fold is unique to the POP gene family and is distant from

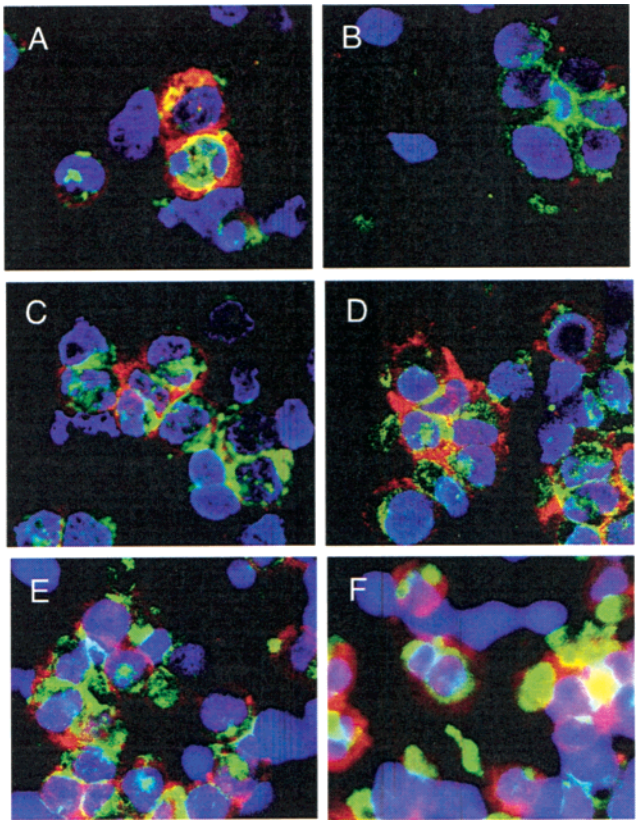

FIGURE 8: Fluorescence microscopy of cytocentrifuged 293T cells. CD26 and its chimeras were stained with a mixture of the anti-CD26 mAbs TA5.9 and B10 followed by Texas Red conjugated goat anti-mouse Ig. Golgi was stained green using wheat germ agglutinin-FITC. The nuclei were stained blue with DAPI. Wild-type CD26 (A), untransfected cells (B), chimeras CD26-DP8 (C), CD26-DP9_4.1 (D), CD26-DP9_4.2 (E), and CD26-DP9_10.3 (F). Representative data from two replicate transfections. Original magnification ×400.

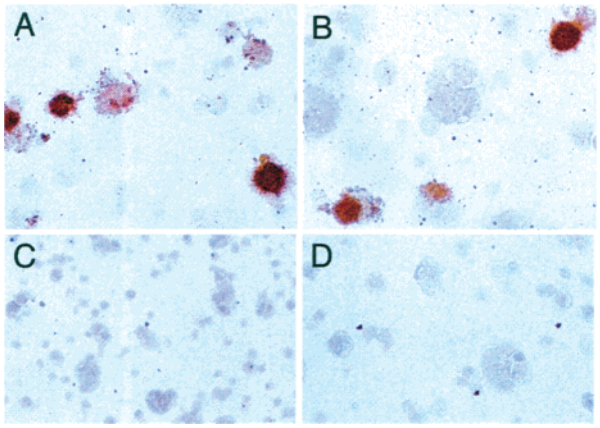

FIGURE 9: Enzyme cytochemistry of cytocentrifuged 293T cells. Hydrolysis of Ala-Pro-4-methoxy-β-naphthylamide-HCl by wild-type CD26 (A) and DP8 (B) produced red staining. CD26-DP8 chimera (C). Untransfected 293T cells (D). Representative data from four replicate transfections. Haematoxylin counter stain. Original magnification ×200 (A, B, and D) and ×100 (C).

the catalytic site. For these reasons it might not directly contribute to catalysis but rather would provide stability to the circular structure of the propeller by covalently linking it to the hydrolase domain (*16*). The absence of enzyme activity and retention of little cell surface expression in these mutants supports this concept. Concordantly, the additional deletion of an N-terminal part of the propeller led to failure to express cell surface CD26 protein, indicating that the predicted propeller blade ending at residues 187 is essential for the biosynthesis of CD26. The physical data reported here concords with our proposed structure of CD26 (3). Truncation of only 20 residues from the CD26 C-terminus ablates enzyme activity (*13*), so the entire α/β hydrolase domain is required for expression of peptidase activity. Further investigations are needed into the mechanisms of dimerization and catalysis.

The recent revival of interest in DPIV inhibitors derives from their potential as therapeutics for controlling type II diabetes (*2*, *4*, *11*, *12*, *41*, *42*). An improved understanding of the CD26-related enzymes and their structure is applicable to this field, and a crystal structure of CD26/DPIV in the presence or absence of an inhibitor would be a significant advance. Our structure−function data indicates that it will be necessary to produce the entire extracellular portion of CD26 rather than isolated domains or fragments for deriving a structure.

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