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Expression, purification, and characterization of human dipeptidyl peptidase IV/CD26 in Sf9 insect cells

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Abstract

The human dipeptidyl peptidase IV/CD26 (DPPIV/CD26) is a multifunctional type-II membrane bound glycoprotein. As a receptor of collagen I and fibronectin it mediates cell–cell and cell–matrix adhesion, and by interacting with extracellular adenosine deaminase and CD45 it is involved in regulatory and costimulatory events in the immune system. DPPIV/CD26 has a very distinct substrate specificity, and is potentially capable of truncating many cytokines, chemokines, and peptide hormones. In this study, we describe the overexpression, purification, and characterization of human DPPIV/CD26 in *Spodoptera frugiperda* (Sf9) cells, using the baculovirus system. Overexpression of DPPIV/CD26 was confirmed by measurement of its peptidase specificity, SDS–PAGE, and Western blot analyses. Expression rates were between 6.4 and 17.6 mg protein per liter suspension culture (1.5×10^9 cells). The N-linked oligosaccharide composition was examined and compared with that of mammalian cell-expressed DPPIV/CD26. Two-step purification by immunoaffinity chromatography and size-exclusion fast protein liquid chromatography (SE-FPLC) led to highly stable protein with significant peptidase activity. A subsequent gel filtration step on a Superdex 200 column yielded 2 mg homogeneous dimeric DPPIV/CD26 (per liter insect cell culture) for crystallographic studies. Protein homogeneity was confirmed by silver staining of non-denaturating PAGE gels and by MALDI-TOF analysis of tryptic peptides.

The multifunctional type-II transmembrane glycoprotein, dipeptidyl peptidase IV (DPPIV, E.C. 3.4.14.5), is expressed in nearly all mammalian tissues. First described as glycyl-prolyl-naphthylamidase by Hopsu-Havu and Glenner [1,2] from rat liver, it has been shown to be identical with the activation marker CD26 of lymphocytes and other immune cells [3–5]. The sequences of DPPIV/CD26-isoforms from human and mouse exhibit 84.9% similarity [6,7]. The wild-type enzyme is expressed as a non-covalently linked 210 kDa homodimer at the cell surface. DPPIV/CD26 consists of a short cytoplasmic domain of six amino acids, followed by a hydrophobic transmembrane domain (aa 7–28) and an extracellular sequence of 739 amino acids [8]. The primary structure contains nine potential N-glycosylation sites, six of them proximate to the trans-membrane domain. The glycan-rich domain is followed by a cysteine-rich region containing 10 of the 12 cysteine residues. The C-terminal active site contains the catalytic triad Ser^{630}, Asp^{708}, His^{740} [9,10].

Besides its well-known exopeptidase activity, DPPIV also exhibits endopeptidase activity towards denatured collagen [11]. Its very distinct substrate specificity is important for the processing and modulation of defined peptides and chemokines [12]. Expression of DPPIV/CD26 is tightly associated with cell adhesion and differentiation [13,14], and it is an important co-stimulant during T-cell activation and proliferation. The specific interaction of DPPIV/CD26 with extracellular adenosine deaminase (ADA) has been shown to significantly modulate immune activity in humans [3,15].

The biological significance of the N-glycans of DPPIV is not fully understood, but correct N-glycosylation of DPPIV/CD26 is important for its biological stability [16]. The cysteine-rich domain of DPPIV, which mediates the binding to ADA or collagen [17,18], contains potential disulfide bridges associated with the functional conformation of the protein [19,20]. The tertiary structure of DPPIV has not been elucidated, but

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initial studies on its determination have been reported [21–23]. To efficiently produce large quantities of homogeneous, enzymatically active protein for crystallography and cryo-electron microscopy, we have constructed recombinant DPPIV/CD26 for overexpression in the baculovirus system. We established a two-step purification protocol for the Sf9-overexpressed DPPIV/CD26 to obtain homogeneous protein samples.

Materials and methods

Materials

Spodoptera frugiperda cells (Sf9 cells), the pFastBacHT transfer vector, competent DH10Bac *Escherichia coli* cells, Sf-900 II SF medium, and CellFECTIN were purchased from Gibco BRL; antibiotics, Bluo-gal, IPTG, and protease inhibitor mix were from Sigma; TNM-FH medium was from Invitrogen; β-octylglucopyranoside was a product of GLYCON Biochem.; Ni-NTA resin and buffers were from Qiagen.

Methods

Production of recombinant baculovirus

The full-length cDNA of DPPIV/CD26 was cloned from a human kidney cDNA bank and ligated into the multiple cloning site of pFastBacHTc (Gibco BRL) vector. Cloning steps were monitored by restriction endonuclease mapping and sequence analysis using the Sanger method [24]. *E. coli* clones with recombinant bacmid were obtained after transformation of *E. coli* DH10Bac cells with 1 ng hCD26/pFastBacHTc plasmid and blue/white-screening according to manufacturer's protocol. Monolayers of Sf9 cells (9×10^5 cells/well in a 6-well plate) were transfected with different amounts of bacmid-DNA using the CellFECTIN reagent (Gibco BRL). The recombinant virus was harvested 72 h post-transfection and further amplified twice to obtain higher titered virus stocks. Virus titers were determined by plaque assay.

Optimization of expression of recombinant protein

Sf9 cells in suspension culture (TNM-FH medium, 10% v/v heat-inactivated FCS, 1% lipid, and 50 U/50 μg penicillin/streptomycin) were infected with recombinant virus with multiplicities of infection (m.o.i.) of 0.7, 1.3, 2.7, and 5.3 plaque-forming units per cell. Cells were harvested 24, 48, and 72 h post-infection, from a 100 ml culture, and solubilized in 5 ml lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 0.7% w/v β-octylglucopyranoside, 1 mmol protease-inhibitor mix (Sigma, P 2714, pH 7.4) by vortexing for 1 h at 4 °C. Cell debris was pelleted by ultra-centrifugation at 100,000*g* for 30 min at 4 °C. The clear supernatant was used directly for immunoaffinity chromatography or stored at −20 °C. To rescue β-octylglucopyranoside-insoluble DPPIV/CD26 from the pellet, resolubilization assays with lysis buffer containing 1% v/v Triton X-100 were performed. This procedure did not result in significantly higher yields of DPPIV/CD26.

Purification of harvested protein by immunoaffinity chromatography

Rabbit anti-CD26 IgGs were separated from polyclonal antiserum by $(NH_4)_2SO_4$ precipitation and covalently coupled to Affi-Gel 10 Gel column according to the protocol of the manufacturer (BioRad). All subsequent purification steps were carried out at 4 °C or on ice. Human DPPIV/CD26 from the supernatant (see above) was bound to the column overnight with a flow rate of 0.5 ml/min. After extensive washing with modified RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.03% w/w SDS, pH 7.2), prewash buffer (10 mM Tris–HCl, 1 M NaCl, pH 7.2), and tris-buffered saline (10 mM Tris–HCl, 150 mM NaCl, pH 7.2), bound protein was eluted with 50 mM ethanolamine, 0.7% w/v β-octylglucopyranoside, pH 10.7, followed by 50 mM ethanolamine, 0.7% w/v β-octylglucopyranoside, pH 11.2. Fractions were collected and promptly neutralized with 1/10 vol. of 0.5 M Tris–HCl, pH 7.3, and concentrated with Centriplus YM-30 centrifugal filters (Millipore) up to 7 mg/ml.

Isolation of homogeneous dimeric DPPIV/CD26 by size-exclusion fast protein liquid chromatography (SE-FPLC)

Four hundred microliters of concentrated elution fractions from immunopurified DPPIV/CD26 was loaded on a Superdex 200 column (Pharmacia Biotech), previously equilibrated with Tris buffer (50 mM Tris–HCl, 0.7% w/v β-octylglucopyranoside, pH 7.4) and eluted at a flow rate of 0.5 ml/min with Tris buffer. The peak corresponding to DPPIV/CD26 appeared after approx. 22.4 min and was confirmed by non-denaturing PAGE [25] and enzyme activity assay. The molecular weight of the eluted protein was determined using thyroglobulin (670 kDa), aldolase (156 kDa), ovalbumin (44 kDa), and cytochrome *c* (17 kDa) (all Pharmacia Biotech) as standards.

Determination of protein concentration and enzymatic activity

The protein concentration was assayed using the BCA Protein Assay Reagent Kit (Pierce). Enzymatic activity was determined with Gly-Pro-4-nitroanilide as substrate as described [26].

Polyacrylamide gel electrophoresis, silver staining, and Western-blot analysis

DPPIV/CD26 purified by immunoaffinity chromatography was subjected to SDS–PAGE according to Laemmli [27]. Homogeneity of concentrated eluates was

tested by non-denaturing PAGE as described by Walker [25]. Separated proteins were transferred onto a nitrocellulose membrane, immunostained with polyclonal antibodies, and visualized by chemiluminescence as described earlier [19].

N-Glycan analysis of insect cell expressed protein by lectin binding assay

The lectin-binding studies were performed using the DIG Glycan Differentiation Kit according to the protocol of the manufacturer (Roche Molecular Diagnostics, Mannheim, Germany). The following lectin–DIG conjugates were applied: GNA (*Galanthus nivalis-agglutinin:* binds to Manα1-2/3/6Man), SNA (*Sambucus niger-agglutinin:* binds to Siaα2-6Gal), MAA (*Maackia amurensis-agglutinin:* binds to Siaα2–3Gal), PNA (*Peanut-agglutinin:* binds to Galβ1-3GalNAc), and DSA (*Datura stramonium-agglutinin:* binds to Galβ1-4GlcNAc).

MALDI-TOF analysis of SE-FPLC purified dimeric DPPIV/CD26

Prior to digestion, SE-FPLC purified dimeric DPPIV/CD26 were treated with 55 mM iodacetamide for 20 min in the dark at room temperature. The protein was then digested with 0.2 μg of trypsin in 50 mM ammonium bicarbonate and 5 mM $CaCl_2$ at 37 °C for 16 h. The mass spectrometric analysis of proteolytically derived peptides was performed using a Bruker Reflex MALDI-TOF mass spectrometer (Bruker, Bremen, Germany) with α-cyano-4-hydroxycinnimanic acid as a matrix.

Results

Overexpression of recombinant human dipeptidyl peptidase IV/CD26

Human dipeptidyl peptidase IV/CD26 cDNA with a N-terminal His_6-tag and a rTEV protease cleavage site was inserted into the baculovirus transfer vector pFastBacHTc. After transposition of the recombinant vector to the baculovirus DNA in *E. coli* and preparation of high titer virus stocks, Sf9 cells were infected with this recombinant baculovirus DNA for protein expression. The DPPIV/CD26 expression level was monitored by measuring the specific peptidase activity. Expression was optimized by variation of the virus titer for infection; the highest specific activity was determined at a m.o.i. = 0.7 (Fig. 1). The time course of enzymatic activity after initial infection was also monitored. Maximum activity was attained 48 h post-infection, while vector-only infected Sf9 cells do not show any enzymatic activity (Fig. 2). For preparative DPPIV/CD26 expression, cells were infected with a m.o.i. of 0.7 and harvested 48 h post-infection. Under these conditions, the DPPIV/CD26 expression rates were between 6.4 and 17.6 mg protein per liter suspension culture (1.5×10^9 cells). His_6-tagged, insect cell-expressed DPPIV/CD26 shows specific enzymatic activity comparable to that of the wild-type enzyme expressed in mammalian cells.

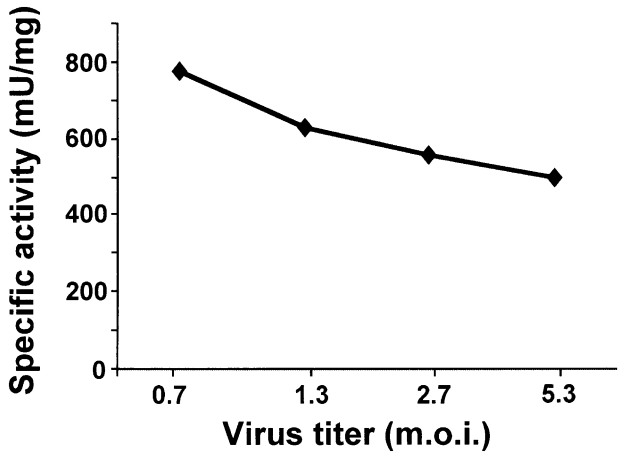

Fig. 1. Protein expression levels at different m.o.i. values. Sf9 Cells were infected with recombinant baculovirus at a m.o.i. of 0.7, 1.3, 2.7, and 5.3. The cells were harvested 72 h post infection.

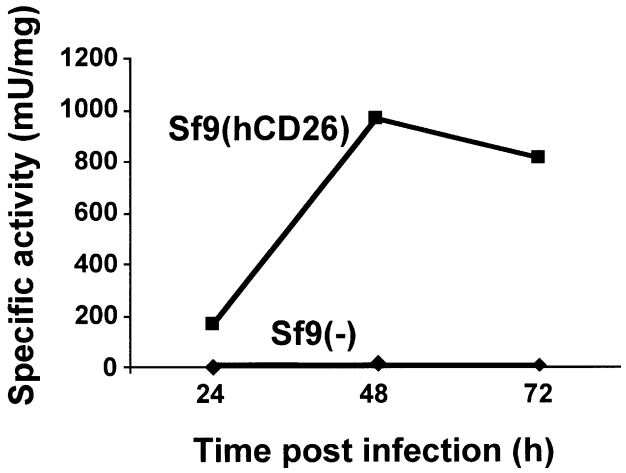

Fig. 2. Time dependence of hCD26 expression in infected Sf9 cells. The cells were infected with the recombinant baculovirus at m.o.i. = 0.7.

Purification of homogeneous, dimeric DPPIV/CD26

The purification of His_6-tagged DPPIV/CD26 by nickel-chelated affinity chromatography yielded poor amounts of protein. From Western blot analysis of the elution fractions we conclude that His_6-binding to the column is very weak and that predominantly the non-active, monomeric form of DPPIV/CD26 is accumulated (data not shown). After generation of polyclonal anti-human DPPIV/CD26 antibodies, the DPPIV/CD26 was purified by immunoaffinity chromatography.

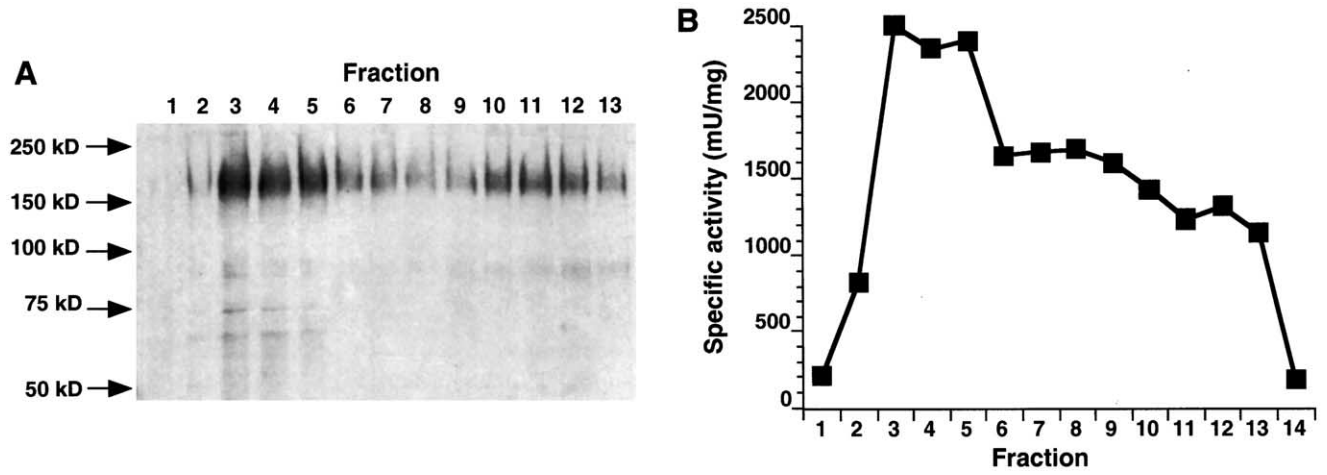

Fig. 3. Analysis of purification of immunoaffinity chromatography harvested human dipeptidyl peptidase IV/CD26. (A) Silver stained non-denaturing PAGE: fractions 1–5: protein eluted with 50 mM diethanolamine, pH 10.7; fractions 6–14: protein eluted with 50 mM diethanolamine, pH 11.2. (B) Specific enzymatic activity of eluted fractions.

Unspecifically bound proteins were removed with three buffers of different ionic strength. DPPIV/CD26 was then eluted with 50 mM ethanolamine. Non-denaturing PAGE followed by silver staining of the gel revealed highly purified, active DPPIV/CD26 (Figs. 3A and B). To isolate dimeric DPPIV/CD26, an additional SE-FPLC gel filtration step was performed. This procedure yielded apparently homogeneous dimeric protein, based on silver stained non-denaturing PAGE (Fig. 4B) and Western blot analyses (Fig. 4C). The purity was further confirmed by MALDI-TOF analysis of peptides derived from tryptic digestion of the protein(s) obtained after SE-FPLC. The detected masses of all peptides matched well with the theoretical masses expected from tryptically digested DPPIV/CD26 (data not shown). The molecular weight of dimeric DPPIV/CD26 is 195 kDa, calculated from the retention time of SE-FPLC (see inset in Fig. 4A). The calculated molecular mass of dimeric DPPIV/CD26, including the peptide backbone, the His_6-tag, and the rTEV-cleavage site, is 174 kDa. The additional 21 kDa are obviously due to oligosaccharide side chains. In the presence of 0.7% w/v β-octylglucopyranoside, which is slightly above the critical micelle concentration, the purified protein could be concentrated up to 7 mg/ml.

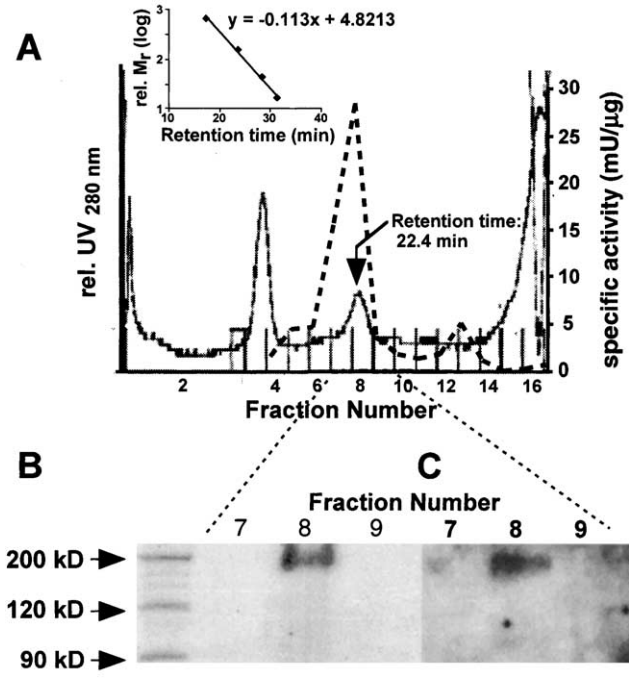

Fig. 4. Isolation of homogeneous, dimeric CD26 by size exclusion chromatography. (A) Typical elution profile of CD26 after size exclusion chromatography on a Superdex 200 column. The standard linear regression curve was generated by plotting the log of the molecular mass of different calibration proteins against their retention times (see inset). Calibration proteins: thyroglobulin (670 kDa), aldolase (156 kDa), ovalbumin (44 kDa), and cytochrome *c* (17 kDa). Elution fractions 7–9 were analyzed by silver staining (B) and Western blotting (C) following non-denaturing PAGE.

Enzymatic activity of purified enzyme

Specific activity of dipeptidyl peptidase IV was used to monitor the expression rate and the purification protocol. Enzymatic activity is tightly associated with the structural integrity of the protein, which in turn depends on correct N-glycosylation [16]. Although the glycosylation pattern in insect cells is different from that in mammalian cells, initial specific activity in Sf9 cell lysate was comparable to the activity found in the lysate of DPPIV/CD26-transfected Chinese hamster ovary cells (data not shown). After optimization of the expression conditions, an initial specific activity of 1088 mU/mg was found in the cleared cell lysate. The

Table 1
Representative purification of human dipeptidyl peptidase IV/CD26 overexpressed in Sf9 cells (100 ml suspension culture, 1.5×10^6 cells/ml)

	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification (fold)
Cleared cell lysate	26.25	28,555	1088	100	1
Immunoaffinity chromatography	0.6	14,825	24,708	51.9	22.7
Concentration step	0.4	11,988	29,970	42	27.5
SE-FPLC	0.2	5661	28,305	19.8	26

activity of immunopurified enzyme was 24,700 mU/mg and increased to 28,305 mU/mg after SE-FPLC gel filtration, i.e., the procedure achieved a total purification factor of 26 with an overall yield of 19.8% corresponding to about 2 mg homogeneous protein from 1 liter of suspension cell culture (Table 1).

Comparison of the lectin-binding patterns of CHO and Sf9 cell-expressed DPPIV/CD26

Purified DPPIV/CD26, expressed in CHO and Sf9 cells, was applied to SDS–PAGE, blotted on a nitrocellulose membrane and incubated with different digoxigenin-conjugated lectins, namely GNA, SNA, MAA, PNA, and DSA. In contrast to CHO cell-expressed DPPIV/CD26, which displayed positive signals with all five used lectins, insect cell-expressed protein solely shows a strong binding to GNA (data not shown), i.e., the predominance of high-mannose structures.

Discussion

The tertiary structure of the multifunctional enzyme dipeptidyl peptidase IV/CD26 has not been elucidated, although physico-chemical investigations implicate a three domain structure of the extracellular part [21]. Molecular modelling studies of the cysteine-rich domain of DPPIV/CD26 on the basis of the crystal structure of prolyl oligopeptidase (15% amino acid identity to DPPIV/CD26) suggest a β-propeller structure for this domain [23]. To conduct an in-depth crystallization study, a large quantity of enzymatically active DPPIV/CD26 is needed. For rapid purification, we chose the His_6-tag overexpression and purification system, which is described for isolation of membrane proteins. Unlike other epitopes, i.e., the GST-tag (25.6 kDa), the His_6-tag is not likely to alter the biological activity of the expressed protein [28,29]. A His_6-tagged protein, which is only 3.2 kDa or 29 amino acids larger (including the rTEV protease cleavage site) than the wild type, was expressed in its dimeric form in Sf9 cells. The enzymatic activity, which is located in the C-terminal domain of DPPIV/CD26, is not influenced by the His-epitope at the N-terminus and is comparable to CHO cell-expressed wild-type DPPIV/CD26 [16,19]. Purification by nickel-chelated affinity chromatography yielded mainly monomeric and oligomeric forms and only small quantities of the desired dimeric DPPIV/CD26. The results indicated that the monomeric form of DPPIV/CD26 was bound to the column and that the protein reassembled predominantly to an unphysiological oligomeric form devoid of enzyme activity after elution (data not shown). To overcome this problem, a polyclonal antiserum against native DPPIV/CD26 was generated and purification by immunoaffinity chromatography was established. The average yield of active DPPIV/CD26 was 80% (Fig. 3). Oligosaccharide side chains of the protein expressed in insect cells consist predominantly of high-mannose structures. It was shown earlier that mannose-rich *N*-glycan chains are sufficient for properly folded and fully active DPPIV/CD26 [30]. Since the purified His_6-tagged construct is slightly more stable than the wild-type enzyme, we did not remove this epitope. For crystallization studies, the existence of a homogeneous protein fraction is essential. Homogeneity was achieved by an additional SE-HPLC gel filtration step (Fig. 4).

With this simple two-step purification protocol, milligram quantities of active human DPPIV/CD26 were prepared for crystallization and cryo-electron microscopy studies.

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