Human Recombinant Pro-dipeptidyl Peptidase I (Cathepsin C) Can Be Activated by Cathepsins L and S but Not by Autocatalytic Processing

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ABSTRACT: Human dipeptidyl peptidase I was expressed in the insect cell/baculovirus system and purified in its active (rhDPPI) and precursor (pro-rhDPPI) forms. RhDPPI was very similar to the purified enzyme (hDPPI) with respect to glycosylation, enzymatic processing, oligomeric structure, CD spectra, and catalytic activity. The precursor, which was a dimer, could be activated ∼2000-fold with papain. Cathepsin L efficiently activated pro-rhDPPI in vitro at pH 4.5 ($k_{app} \sim 2 \times 10^3$ min^{-1} M^{-1}), and two cleavage pathways were characterized. The initial cleavage was within the pro region between the residual pro part and the activation peptide. Subsequently, the activation peptide was cleaved from the catalytic region, and the latter was cleaved into the heavy and light chains. Alternatively, the pro region was first separated from the catalytic region. Cathepsin S was a less efficient activating enzyme. Cathepsin B and rhDPPI did not activate pro-rhDPPI, and the proenzyme was incapable of autoactivation. Incubation of both pro-rhDPPI and rhDPPI with cathepsin D resulted in degradation. Cystatin C and stefins A and B inhibited rhDPPI with K_i values in the nanomolar range ($K_i = 0.5-1.1$ nM). The results suggest that cathepsin L could be an important activator of DPPI in vivo and that cathepsin D and possibly the cystatins may contribute to DPPI downregulation.

Dipeptidyl peptidase I (DPPI,[1] cathepsin C) is a lysosomal dipeptidyl aminopeptidase belonging to the papain superfamily of cysteine peptidases (*1*−*3*). The enzyme is constitutively expressed in many tissues with the highest levels in lung, kidney, liver, spleen, and placenta (*3*−*6*).

In addition to its role in the lysosomal protein degradation, DPPI functions as a key enzyme in the activation of granule serine peptidases in cytotoxic T lymphocytes and natural killer cells (granzymes A and B), mast cells (tryptase and chymase), and neutrophils (cathepsin G and elastase). These peptidases function in cell-mediated apoptosis, inflammation, connective tissue remodeling, etc. (*6*−*10*). To prevent undesired degradation within the host cells, they are translocated to the granules as inactive zymogens with N-terminal activation dipeptides. The role of DPPI is to remove these dipeptides, probably within the granules. DPPI has been demonstrated to be capable of activating the zymogens of tryptase (*11*), chymase (*12*, *13*), cathepsin G (*14*), and granzymes A (*15*), B (*16*), and K (*17*) in vitro. Granzymes A and B isolated from the granules of cytotoxic lymphocytes of DPPI −/− mice had retained their activation dipeptides and were inactive (*18*), indicating that DPPI is essential for granzyme activation also in vivo. Furthermore, two groups independently identified deleterious mutations in the *CTSC* gene encoding human DPPI (hDPPI) as the causes of development of the autosomal recessive disorder Papillon−Lefèvre syndrome (PLS) mainly characterized by severe early onset periodontitis and palmar plantar keratosis (*19*, *20*).

The cDNA sequences encoding rat, human, murine, dog, bovine, and two *Schistosoma* DPPIs have been described and encode preproenzymes (prepro-DPPI), each consisting of a signal peptide, a pro region, and a catalytic region (*2*). The amino acid sequences of the catalytic regions are homologous to the sequences of mature papain family peptidases, including cathepsins L, S, B, H, and K (*2*).

The DPPI zymogens are translocated from the endoplasmic reticulum to the lysosomes by the mannose 6-phosphate pathway and are extensively processed (*21*). Mature enzyme consists of the N-terminal part of the pro region (residual pro part) and of the catalytic region (*22*−*24*). The C-terminal part of the pro region functions as an activation peptide and is liberated from the enzyme during maturation. The catalytic part is cleaved into a heavy and a light chain of 164 and 69 residues, respectively (hDPPI), and the active enzyme exists as a tetramer (*22*). In contrast, the other members of the papain family are monomers, and only cathepsin H retains a small part of its propeptide (the minichain) in the mature form (*25*).

Proteolytic maturation of various cathepsins from the papain family has previously been studied, and papain (*26*) and cathepsins B (*27*, *28*), K (*29*), L (*30*−*32*), S (*33*, *34*), and F (*35*) are all capable of autoactivation. However, the

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[1] Abbreviations: 4-MeNA, 4-methoxynaphthylamide; DPPI, dipeptidyl peptidase I; hDPPI, native human DPPI; GlcNAc, *N*-acetylglucosamine; pNA, *p*-nitroanilide; rhDPPI, recombinant human DPPI; TFA, trifluoroacetic acid.

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activation process of pro-DPPI has not been characterized. In light of the acknowledged regulatory role of DPPI in the activation of granule serine peptidase zymogens and potential functions of DPPI released from mast cells upon degranulation (*36*−*38*), activation and inhibition of human DPPI have been investigated using native and recombinant human DPPI and recombinant human pro-DPPI.

EXPERIMENTAL PROCEDURES

Materials. E-64, *p*-nitrophenyl *p*′-guanidinobenzoate and the DPPI substrates Gly-Phe-*p*-nitroanilide (pNA) and Gly-Arg-pNA were from Sigma. Crystalline papain and Pefabloc SC were from Boehringer Mannheim (Germany) and succinyl-Ala-Ala-Pro-Leu-pNA, succinyl-Ala-Ala-Pro-Arg-pNA, and Gly-Phe-4-methoxynaphthylamide (4-MeNA) from Bachem (Bubbendorf, Switzerland).

Proteins. Chicken cystatin and bovine cathepsin D used for activation of pro-rhDPPI were from Sigma. Bovine trypsin (TPCK-treated) and chymotrypsin (TLCK-treated) for fragmentation of rhDPPI chains were from Worthington (Lakewood, NJ). Endoglycosidase H was from Invitrogen (Carlsbad, CA). The concentrations of cathepsin D and chicken cystatin were estimated on the basis of the manufacturers' specifications. Recombinant human cathepsins B, L, and S were produced as previously described (*34*, *39*, *40*) and their concentrations determined by active site titration with E-64. Recombinant human stefins A and B and recombinant human cystatin C were prepared as described previously (*41*, *42*). Papain, which was affinity purified (*43*) and titrated with E-64, was used for titration of the inhibitors (*44*).

Production of Native and Recombinant Human DPPI and Variants. Native human DPPI (hDPPI) was purified from kidney as previously described (*45*). Active site-directed mutagenesis of the human pro-hDPPI cDNA (*2*) was performed by the two-step PCR procedure reported by Nelson and Long (*46*) or by direct PCR amplification using mutagenic oligonucleotides that spanned the unique *Sac*I or *Bsu*36I restrictions sites (T7A and S254A variants). *Sac*I−*Eco*RI (T7A, S31A, and T97A variants) or *Eco*RI−*Bsu*36I (C234S and S254A variants) fragments of wild-type pro-hDPPI cDNA were subsequently replaced with the appropriate mutagenized cDNA fragments.

Wild-type and variant forms of human pro-DPPI (*2*) fused to the signal peptide from rat DPPI were expressed in the baculovirus/insect cell system and purified from the medium according to the protocols developed for rat DPPI (*47*). Briefly, the different forms of recombinant human DPPI (rhDPPI) were expressed as secreted proteins in High Five cells after infection with recombinant viruses at multiplicities of infections of 5−10. The cultures were harvested 5 days postinfection, and rhDPPI was purified from the medium on butyl-Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden) at pH 4.5. The pool obtained was incubated for 1 or 2 days at pH 4.5 to complete the proteolytic maturation process. After incubation, rhDPPI was desalted and subjected to cation exchange on Q-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) to further purify and concentrate the preparation.

The pro forms of rhDPPI (wild type and inactive C234S variant abbreviated pro-rhDPPI and pro-rhDPPI^{Cys}, respectively) were expressed by use of similar baculovirus constructs and purified by the same overall procedure as applied for mature rhDPPI. However, to minimize proteolytic processing in the culture medium, cells were harvested 2 days earlier (on day 3), and the purification was performed at pH 7.0 throughout the procedure. Pro-rhDPPI did not bind to the Q-Sepharose column in the final purification step but was found in the flow-through in pure form. Recombinant hDPPI was quantified using an extinction coefficient at 280 nm of 2.0.

Amino Acid Sequencing. Amino acid sequencing was carried out in Applied Biosystems 476A or 494A protein sequencers operated according to the manufacturer's instructions. All cysteine residues indicated were positively identified either as alkylated cysteines or as the acrylamide adduct.

HPLC Purification. HPLC purifications of rhDPPI preparations were done on a Symmetry C_8 reversed-phase column (2.1 × 50 mm) (Waters, Milford, MA) using linear gradients of acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA). Peptides generated by enzymatic digestions of rhDPPI heavy and light chains were separated using a Symmetry C_{18} reversed-phase column (2.1 × 50 mm) (Waters, Milford, MA) using linear gradients of acetonitrile in 0.1% aqueous TFA.

Enzymatic Peptide Fragmentation. Before degradation of rhDPPI peptides with either trypsin or chymotrypsin, HPLC-purified heavy and light chains were denatured, reduced, alkylated, and desalted. Lyophilized samples were denatured in 300 μL of 6 M guanidinium chloride and 0.3 M Tris-HCl, pH 8.3, and incubated overnight at 37 °C before addition of 100 μL of 0.1 M DTT in the same buffer. Following 30 min incubation at 22 °C to reduce the disulfide bonds, 100 μL of 0.6 M iodoacetamide in denaturation buffer was added, and the free thiol groups were alkylated for 30 min (22 °C). Finally, samples were desalted into 50 mM NH_4HCO_3 on NAP-5 columns (Pharmacia, Uppsala, Sweden). Enzymatic degradations of the rhDPPI peptides were carried out for 16 h at 37 °C in a thermomixer using either 5 μg (light chain) or 10 μg (heavy chain) of enzyme.

MALDI-TOF Mass Spectrometry. MALDI-TOF mass spectrometry was done using a Bruker Reflex III instrument operated in linear mode for proteins and in reflector mode for peptides. Sample preparation for MALDI-TOF mass spectrometry consisted of mixing 5 μL of sample with 5 μL of 69.9% H_2O/30% acetonitrile/0.1% TFA before addition of 10 μL of matrix solution. The matrix solution was a saturated solution of α-cyano-4-hydroxycinnamic acid in 69.9% H_2O/30% acetonitrile/0.1% TFA. Mass spectra were calibrated externally with proteins and peptides of known masses.

Gel Filtration Chromatography. The oligomeric structures of rhDPPI, pro-rhDPPI, and pro-rhDPPI^{Cys} were determined by gel filtration analysis on an analytical Superdex 200 HR 10/30 column (Pharmacia, Uppsala, Sweden) in 20 mM sodium phosphate, pH 7.0, and 500 mM NaCl and at a flow rate of 0.7 mL/min. Catalase, aldolase, His-tagged pyroglutamyl peptidase I, bovine serum albumin, ovalbumin, and lysozyme were used as standards.

Circular Dichroism Studies. Circular dichroism was measured at 25 °C with an AVIV 62A DS circular dichroism spectrometer (Lakewood, NJ). Cells with path lengths of 1 cm (near-UV region, 320−250 nm) or 0.1 cm (far-UV region, 250−200 nm) were used. Prior to measurements, the

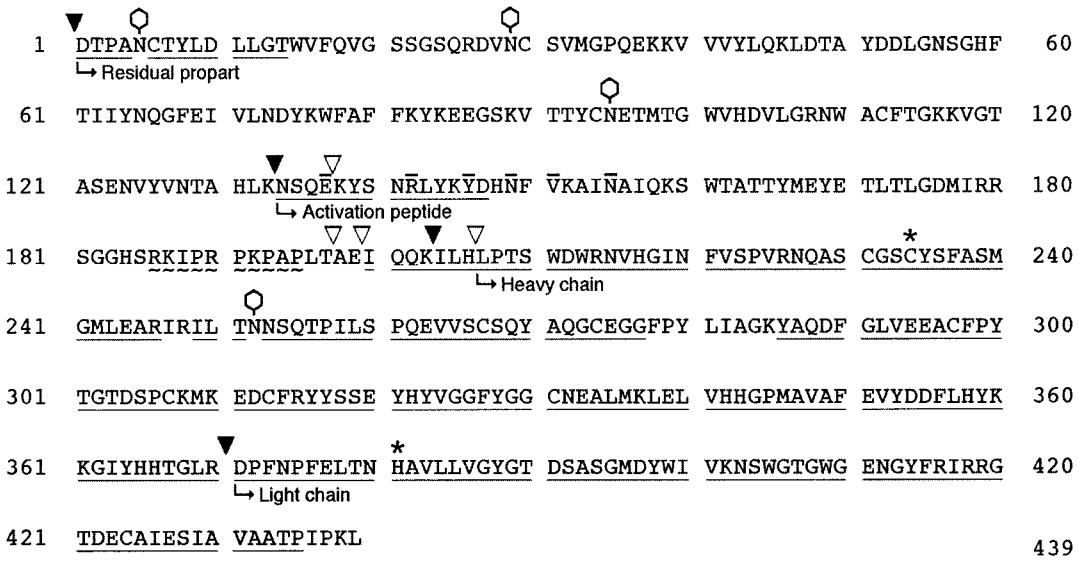

FIGURE 1: Amino acid sequence of human DPPI. The native N-termini of the residual pro part and of the heavy and light chains and the N-terminus of the rhDPPI activation peptide following cathepsin L cleavage (see below) are indicated by bent arrows. The major signal peptidase and cathepsin L processing sites in rhDPPI are indicated by filled arrowheads. Minor or secondary processing sites are indicated by open arrowheads. N-Glycosylated rhDPPI residues are marked with hexagons and the catalytic Cys and His residues by asterisks. Residues in the ERFNIN and PRK motifs (see Discussion) are overlined and underlined with ∼, respectively. The amino acid residues sequenced are underlined. Amino acid residues are numbered from the N-terminus of the proenzyme.

enzymes were dialyzed against 50 mM phosphate buffer, pH 6.0, containing 0.5 mM EDTA.

In Vitro Maturation of rhDPPI Pro Forms. Pro forms of rhDPPI were treated with cathepsins B, L, and S in 20 mM citric acid, 150 mM NaCl, 1 mM EDTA, and 10 mM DTT, pH 4.5 or 4.5−6.9 (cathepsin B only). The buffer used for cathepsin D treatment was 20 mM citric acid, pH 4.5, and 150 mM NaCl. All activation experiments were performed at 22 °C.

Cathepsin D Inactivation of rhDPPI. Cathepsin D (0−30 μM) was incubated with 0.7 μM (0.1 mg/mL) rhDPPI in 50 mM citric acid, pH 5.0, and 150 mM NaCl (22 °C). Proteolytic degradation of DPPI was followed by measurement of residual DPPI activity and by SDS−PAGE.

Kinetic Studies. DPPI activities were followed in 20 mM citric acid, pH 4.5, and 150 mM NaCl using Gly-Phe-pNA as substrate (*47*) or in 20 mM sodium phosphate, pH 7.0, and 150 mM NaCl using Gly-Arg-pNA as substrate. Kinetics of hydrolysis of Gly-Phe-4-MeNA by native and recombinant DPPI, active site titrated with cystatin C (*45*), were investigated in 100 mM sodium phosphate, pH 6.0, containing 1 mM EDTA and 1 mM dithiothreitol as described (*22*). Inhibition studies with stefins A and B and cystatin C were performed in the same buffer using 20 μM Gly-Phe-4-MeNA as substrate essentially as described elsewhere (*42*, *45*, *48*). Endopeptidase activities were assayed with 0.5 mM suc-Ala-Ala-Pro-Leu-pNA and suc-Ala-Ala-Pro-Arg-pNA in 20 mM citric acid, pH 4.5, and 150 mM NaCl.

RESULTS

Production of Pro-rhDPPI and Mature rhDPPI. Pro-rhDPPI was isolated from the medium on day 3 at pH 7.0 to prevent processing of the zymogen. Pro-rhDPPI did not bind to the Q-Sepharose column, which was therefore used as an efficient tool to remove contaminating proteins, including mature rhDPPI. Typically, 20−30 mg of mature rhDPPI was purified per liter of culture. Because of the early time of harvest of pro-rhDPPI, only 10−15 mg/L pure pro-hDPPI was isolated. The specific activity of purified pro-rhDPPI could be increased ∼2000-fold by activation with papain (*47*).

Primary Structures. Following HPLC fractionation of the rhDPPI peptides, the four main fractions were analyzed by MALDI-TOF mass spectrometry. Two of these fractions contained a heterogeneous component (∼19.9 kDa) which was subsequently identified as the heavy chain by the N-terminal amino sequence I^{204}LHLPT... (Figure 1). Approximately 20% of the heavy chain peptides started at L207, which is the N-terminus of the purified hDPPI heavy chain (*2*, *22*). The calculated protein mass of the rhDPPI heavy chain is 18829.3 Da (residues 204−370), indicating that the rhDPPI heavy chain was glycosylated.

The third fraction contained a component with a mass of 7581.0 Da, which is in agreement with the theoretical mass of the hDPPI light chain (7577.5 Da; residues 371−439). The identity of the peptide was confirmed by amino acid sequencing of the 49 N-terminal amino acid residues (Figure 1).

HPLC-purified heavy and light chains were degraded with trypsin and selected peptides sequenced. As shown in Figure 1, a total of 94% of the amino acid residues of rhDPPI heavy and light chains were sequenced. The C-terminus of the heavy chain was verified experimentally, demonstrating that the heavy and light chains were separated by a single, specific cleavage between R370 and D371.

The fourth fraction of HPLC-purified rhDPPI peptides contained both the light chain and a highly heterogeneous component with masses between 16.5 and 19 kDa. The presence of peaks with spacings of ∼162 Da within the broad peak is indicative of heterogeneous glycosylation, as 162 Da is the mass of hexose residues. This component was presumably the residual pro part of rhDPPI. The finding of the three rhDPPI chains in different fractions following inactivation of reducing agents with TFA (pH 2) showed

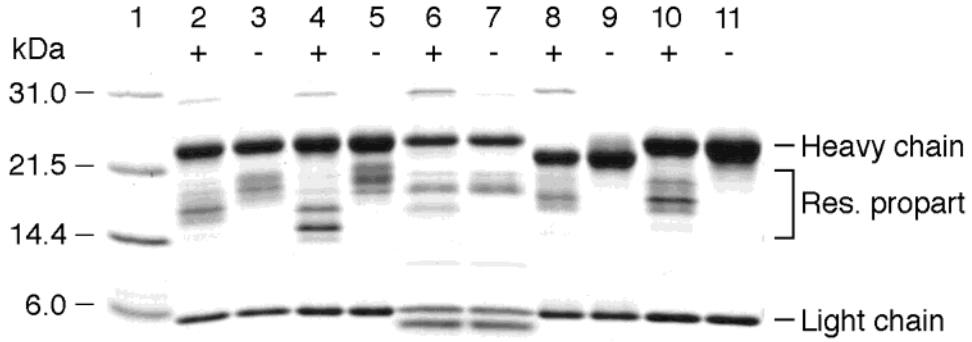

FIGURE 2: Deglycosylation of rhDPPI wild type and single site N-glycosylation variants. The potential residual pro part glycosylation sites at N5 (lanes 2 and 3), N29 (lanes 4 and 5), and N95 (lanes 6 and 7) and the single heavy chain site at N252 (lanes 8 and 9) were eliminated and the purified variants and wild-type rhDPPI (lanes 10 and 11) analyzed by SDS−PAGE after treatment with (+) or without (−) endoglycosidase H as indicated. Only the variant with the eliminated glycosylation site at N95 was insensitive to endoglycosidase H treatment, showing that only the glycosylation at this position was affected by the enzyme. The 5 kDa band observed in lanes 6 and 7 is the result of partial proteolytic degradation of the light chain during activation. Molecular mass markers are shown in lane 1.

that the peptides were not covalently linked under storage conditions in the presence of 2 mM cysteamine. No (*24*) or some (*22*) disulfide linkage of the individual chains has been reported for native hDPPI. To determine the N-terminus of the residual pro part, endo H-deglycosylated rhDPPI was subjected to SDS−PAGE and electroblotting, followed by amino acid sequencing (*47*). A single amino acid sequence (D^1TPAXX^6), which corresponds to the N-terminal sequence of the residual pro part (*22*), was obtained. The two unidentified residues correspond to a glycosylated asparagine and an oxidized cysteine, respectively.

rhDPPI Glycosylation. To reveal the level of rhDPPI glycosylation, each of the four potential N-glycosylation sites were individually destroyed by substituting the required Ser or Thr residue in the consensus sequence for N-glycosylation with an Ala residue (-N-X-S/T- to -N-X-A-). The results of SDS−PAGE analysis of the purified variants showed that all four potential sites (N5, N29, and N95 in the propeptide and N252 in the heavy chain) were glycosylated (Figure 2) as in the native enzyme (*24*). Compared to wild-type rhDPPI, the N5 and N29 glycosylation site variants showed increased residual pro part and unchanged heavy chain mobilities. The apparent molecular mass of both residual pro parts was further reduced by endoglycosidase H treatment. Similarly, deletion of the third site at N95 affected the migration and homogeneity of the residual pro part. However, no further mass reduction was observed following endoglycosidase H treatment. Finally, elimination of the site at N252 resulted in an increased heavy chain mobility. The residual pro parts of this variant and of the wild-type rhDPPI displayed the same migration pattern following endoglycosidase H treatment (Figure 2).

The glycosylation on N252 in the heavy chain was further investigated. The rhDPPI heavy chain was digested with chymotrypsin, and an HPLC-purified peptide exhibiting mass heterogeneity was completely sequenced ($T^{251}-Y^{270}$). The experimental masses of the heterogeneous peptide exceeded the calculated protein mass, and the differences of 896 and 1057 Da correspond well to the masses of carbohydrate structures consisting of two *N*-acetylglucosamine (GlcNAc) and three hexoses (892 Da) and of two GlcNAc and four hexoses (1054 Da). In addition, the mass differences correspond to the mass difference of 1.0−1.1 kDa observed between the theoretical mass of the intact rhDPPI heavy chain (18 829.3 Da) and the mass measured (19.9 kDa). MALDI-TOF mass spectrometry of the rhDPPI heavy chain without glycosylation on N252 (isolated from the S254A variant) gave a mass of 18 827 Da. Accordingly, the heavy chain does not carry other modifications giving rise to major mass differences.

Oligomeric Structures. The oligomeric states of pro-rhDPPI, pro-rhDPPI^{Cys}, and rhDPPI were investigated by gel filtration chromatography. The proenzymes migrated with indistinguishable elution volumes corresponding to a mass of 77 kDa whereas rhDPPI migrated with a mass of 140 kDa. The results suggest that both proenzymes are dimers and that rhDPPI is a tetramer, in agreement with previous results (*21*, *22*).

Circular Dichroism Measurements. Both the far- and near-UV CD spectra of hDPPI and rhDPPI (Figure 3) were very similar, confirming that the native and recombinant forms have the same content of secondary structural elements and that the aromatic residues are located in very similar environments. However, the spectra of pro-rhDPPI^{Cys} differed substantially from those of the mature proteins, reflecting the contribution of the activation peptide.

Catalytic Activity of hDPPI and rhDPPI. On the basis of active site titrations with cystatin C, rhDPPI was ∼85% active, whereas the activity of hDPPI was ∼50%. Values of K_m for Gly-Phe-4-MeNA were similar [(65−75) ± 7 μM] whereas the k_{cat} value for rhDPPI (750 ± 60 s^{-1}) was five times that for hDPPI (150 ± 12 s^{-1}). The different k_{cat} values probably reflect differences in glycosylation and processing between the two enzyme variants, although some effect of catalyticaly inactive material complexed by cystatin C in the titration experiment cannot be excluded.

Proteolytic Activation of rhDPPI Pro Forms. To determine if the processing was autocatalytic, the active site cysteine was mutated to an serine (C234S) and the pro form of the variant (pro-rhDPPI^{Cys}) produced. Pro-rhDPPI^{Cys} purified on butyl-Sepharose at pH 7.0 and incubated at pH 4.5 was processed. In contrast, proenzyme which was further purified by a passage through Q-Sepharose showed no sign of processing (Figure 4), indicating that the processing was caused by a contaminant peptidase and was not dependent on DPPI activity.

Incubation of semipurified pro-rhDPPI (butyl-Sepharose step only) for 22 h at pH 4.5 and 22 °C with cysteine

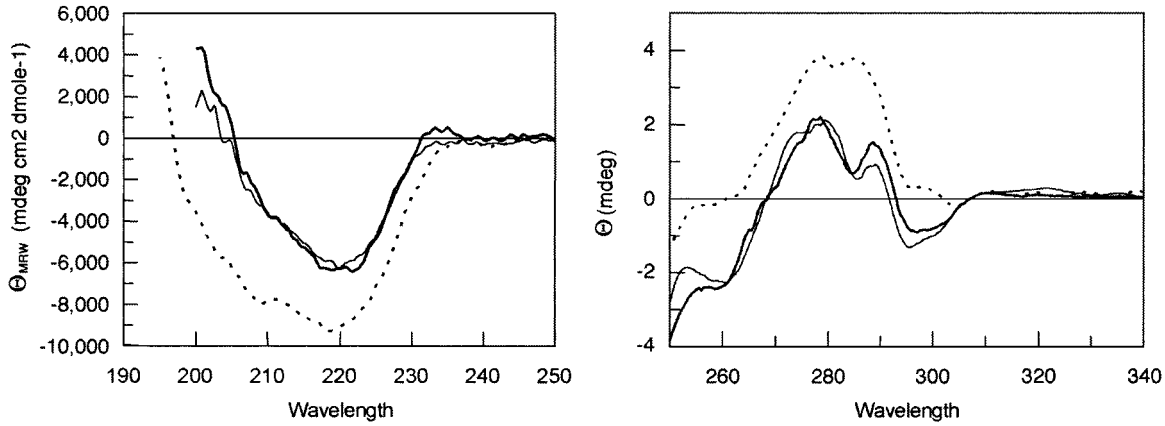

FIGURE 3: CD spectra of hDPPI, rhDPPI, and pro-rhDPPI. The far-UV (left) and near-UV (right) spectra of hDPPI (thick lines) and rhDPPI (thin lines) were determined to estimate the structural similarity of the native and recombinant enzymes. The spectra are highly similar but differ significantly from those of pro-rhDPPI (broken lines), which possesses the activation peptide.

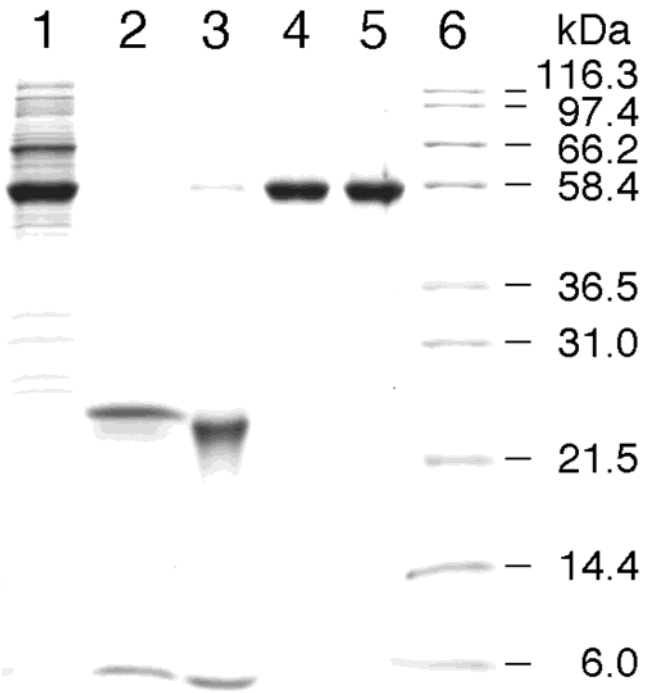

FIGURE 4: Activation of partially purified and pure pro-rhDPPI^{Cys} at pH 7.0 and 4.5. The proenzyme C234S variant isolated after chromatography on butyl-Sepharose (lanes 1 and 2) or after further purification by passage through Q-Sepharose (lanes 4 and 5) was incubated for 18 h at 4 °C and pH 7.0 (lanes 1 and 4) or pH 4.5 (lanes 2 and 5). The same results were obtained with the wild-type enzyme. A rhDPPI standard is shown in lane 3, and molecular mass markers are shown in lane 6.

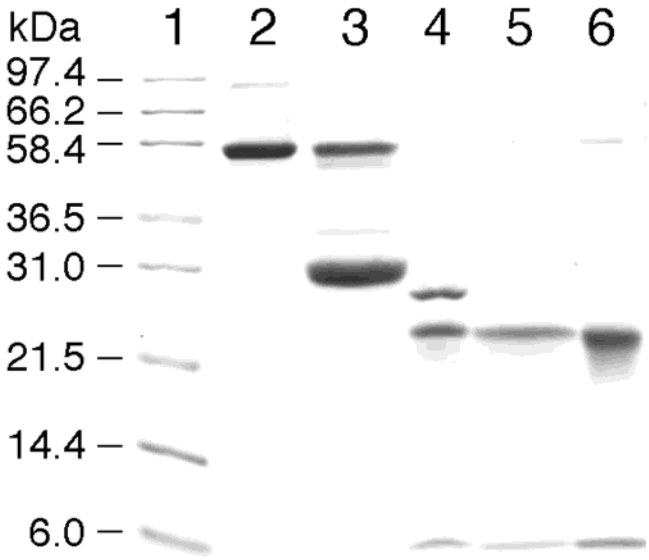

FIGURE 5: SDS−PAGE analysis of pro-rhDPPI following treatment with cathepsins B, S, and L. Purified pro-rhDPPI was incubated with cathepsins B (lane 3), S (lane 4), and L (lane 5) at pH 4.5 and 22 °C for 22 h, and the reactions were stopped by the addition of SDS−PAGE sample buffer. The initial concentration of pro-rhDPPI (lane 2) was 5 μM, and the concentrations of active cathepsins B (band at 31 kDa), S (band at 27 kDa), and L were 5, 1, and 0.2 μM, respectively. Molecular mass markers and the rhDPPI control are shown in lanes 1 and 6, respectively.

peptidase inhibitors E-64 (1 μM) and chicken cystatin (0.1 μM) completely abolished activation, whereas metallopeptidase (1 mM EDTA) and serine (2 mM Pefabloc SC) peptidase inhibitors had no inhibitory effect (data not shown), indicating that the processing enzyme was a cysteine peptidase with the baculovirus-encoded viral cathepsin (v-cath) peptidase being a likely candidate (*49*).

Activation of Pro-rhDPPI by Cathepsins B, L, S, and D. The abilities of the human lysosomal cysteine peptidases cathepsins B, L, and S, of mature rhDPPI, and of the lysosomal aspartic peptidase cathepsin D to process and activate pro-rhDPPI were investigated. Cathepsins L and S processed the wild-type proenzyme into active rhDPPI. By contrast, cathepsin B showed little or no processing of pro-rhDPPI at pH 4.5 (Figures 5 and 6), pH 5.3, 6.1, and 6.9 (data not shown). Incubation of pro-rhDPPI with mature rhDPPI also had no effect whereas incubation of the precursor with cathepsin D resulted in degradation and not activation, as judged on the basis of SDS−PAGE analysis and substrate hydrolysis (results not shown).

The cathepsin L catalyzed reaction was investigated in further detail (Figure 7), and the resulting main components were identified by N-terminal amino acid sequencing following electroblotting. Maturation of pro-rhDPPI (apparent mass of 58 kDa) was initiated by the release of the residual pro part (22−24 kDa, N-terminal sequence D^{1}TPAXCTYLDLLGT^{14}) and accumulation of a 36 kDa peptide (N^{134}SQEKYSNRLYKYD^{147}) composed of the activation peptide and of the heavy and light chains (Figure 1). The N-terminal sequence of the 36 kDa peptide indicates cathepsin L cleavage after K^{133}. In one sample, however, the sequence was found to be K^{138}YSNRL^{143}, which probably

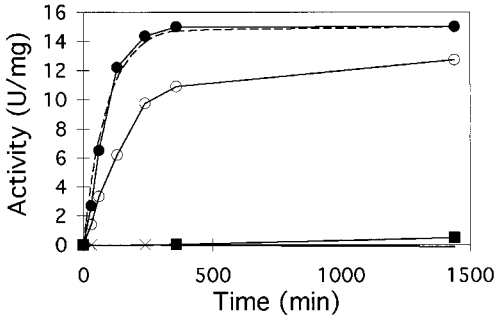

FIGURE 6: DPPI activity during maturation of pro-rhDPPI. Purified pro-rhDPPI was incubated with cathepsins L (●), S (○), or B (■) or without peptidase (×) under conditions identical to those described in the legend to Figure 5. The apparent second-order rate constant for the cathepsin L catalyzed reaction was 2×10^3 min^{-1} M^{-1}, and the calculated progress curve is shown (broken line).

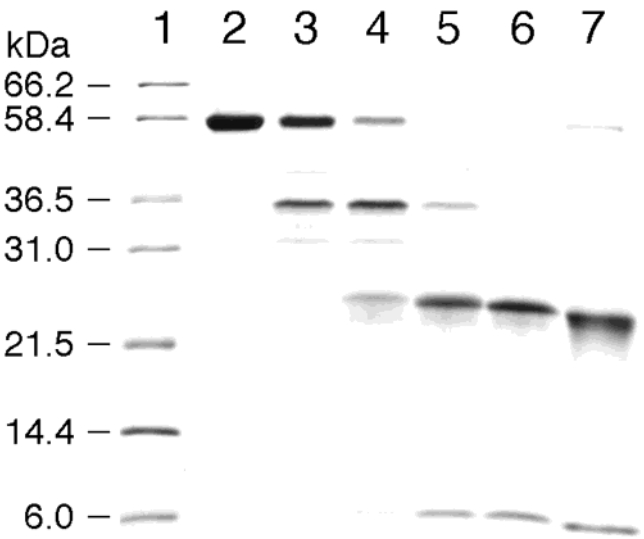

FIGURE 7: SDS−PAGE of pro-rhDPPI maturation by cathepsin L. The conditions were identical to those described in the legend to Figure 5. The progress of the maturation reaction was analyzed after 0.5 h (lane 3), 1.5 h (lane 4), 4 h (lane 5), and 24 h (lane 6). Controls of pro-rhDPPI and rhDPPI are shown in lanes 2 and 7, respectively. Molecular mass markers are shown in lane 1. See text for further details.

resulted from subsequent excision of the dipeptides $N^{134}S^{135}$ and $Q^{136}E^{137}$ by rhDPPI itself. The cathepsin L cleavage site after K^{133} is located 14 residues C-terminal to the cleavage site after G^{119} reported by Cigic et al. (*24*). The investigations did not show if pro-rhDPPI was also cleaved at or close to G^{119} before or after the cleavage at K^{133}. However, an electron density map obtained by X-ray crystallographic analysis of (v-cath processed) rhDPPI (unpublished results) only defines the residual pro-part residues Asp^1−Gly^{119}, corroborating that Gly^{119} constitutes the natural C-terminus of this peptide. Further processing resulted in the separation of the heavy chain (24 kDa, I^{204}LHLPTSXDXRNVHGIN^{220}) and the light chain (6 kDa, D^{371}PFNPFELTNHAVLL^{385}).

During cathepsin L processing of pro-rhDPPI, minor amounts of peptides with apparent masses of 40, 33, and 31 kDa were observed (Figure 7) in addition to the main components described above. The 31 kDa (I^{200}QQKILHLPTSWD^{212}) and 33 kDa (D^1TPAXCTYLDLLG^{13}) peptides resulted from alternative processing of pro-rhDPPI initiated by cleavage between the pro region and the catalytic domain (Figure 1). The sequence of the 31 kDa peptide indicates cathepsin L cleavage after T^{197} and subsequent excision of the $A^{198}E^{199}$ dipeptide by active rhDPPI. Later in the processing, at least residues I^{200}QQK^{203} are removed. Finally, the 40 kDa peptide (ATLTFDHSLEAQ) was identified as procathepsin L, contained in the preparation of cathepsin L.

Modulation of DPPI Activity. The interactions of rhDPPI with cathepsin D and with intracellular (stefins A and B) and extracellular (cystatin C) inhibitors were investigated. Incubation of 0.7 μM rhDPPI with 0−30 μM cathepsin D, which degraded pro-rhDPPI, resulted in progressive inactivation ($k_{app} \sim 4$ M^{-1} s^{-1}). In the presence of 30 μM cathepsin D, the half-life of rhDPPI was about 2 h whereas the activity of rhDPPI incubated in the absence of cathepsin D did not change over a period of 3 h. Degradation of rhDPPI by cathepsin D was verified also by SDS−PAGE (results not shown).

The equilibrium dissociation constants determined with stefins A and B were 1.1 and 0.7 nM, respectively, and cystatin C inhibited rhDPPI with a K_i value of 0.5 nM. The latter value is in good agreement with the dissociation constants of 0.22 nM reported for the interactions of chicken cystatin with both native human (*45*) and bovine (*50*) DPPI.

DISCUSSION

Recombinant human DPPI and pro-DPPI were expressed and isolated in order to characterize the proteolytic maturation of DPPI and its interaction with potential regulatory proteins. As in hDPPI, the recombinant enzyme was fully N-glycosylated (*24*), and disulfide bridges did not covalently link the chains (*22*, *24*). The proteolytic processing was, however, found to be slightly different. The heavy chain was longer at the N-terminus by three amino acid residues, and the residual pro part possibly carried a C-terminal extension. The additional residues at the N-terminus of the heavy chains resemble the extensions observed in dog mast cell DPPI (*37*), in recombinant rat DPPI (*47*), and in cathepsins B (*27*), K (*29*), and L (*32*). They did not substantially affect the catalytic activity of the enzyme, the interaction with cystatins, or formation of active tetramers. Also, the CD spectra of native and recombinant hDPPI and rhDPPI were closely similar. The recombinant hDPPI was thus very similar to purified hDPPI, providing a solid basis for further studies.

The activation process involving activation peptide excision and dimer to tetramer conversion was investigated using purified pro-rhDPPI and rhDPPI. The zymogen was shown to have different far- and near-UV CD spectra from rhDPPI. A further difference was the dimeric structure of pro-rhDPPI. This contrasts with the tetrameric structure of rhDPPI but is in accord with the existence of hDPPI zymogen dimers in vivo (*21*). The fact that DPPI is unique among lysosomal cysteine peptidases in retaining a significant part of the pro region and in forming oligomers in the mature form strongly suggests that the residual pro part is essential in oligomerization.

At the sequence level, the zymogen differs from the active form by the presence of the activation peptide. Recently, the mammalian DPPI activation peptide sequences were suggested to be homologous to the propeptide sequences of the papain-like peptidases (*51*) on the basis of the presence of the conserved ERFNIN motif (*52*, *53*) (Figure 1). In addition,

a region rich in proline and/or basic residues (PRK region) is present in DPPI activation peptides (Figure 1) and in the propeptides of the homologous cathepsins (e.g., residues R^{81p}KPRKGK^{87p} in human procathepsin L and residues P^{49p}KPPQR^{54p} in human procathepsin B). However, the activation peptide of DPPI seems to play a more complex role in the activation process than the propeptides of related cathepsins. Besides its inhibitory function and unknown interaction with the residual pro part, it also prevented the assembly of zymogen dimers to tetramers. This is probably due to the localization of the activation peptides in the dimer surface region that forms the dimer−dimer interface in the active tetramer.

In contrast to most other cathepsins (*26*−*35*), pro-rhDPPI was not capable of autoactivation. Addition of rhDPPI did not activate the zymogen. This is consistent with nonautocatalytic processing of procathepsin X, which is also an exopeptidase (*54*). Three lysosomal cysteine endopeptidases were therefore tested as potential in vivo activators of DPPI, and cathepsins L and S efficiently activated the enzyme. The processing of pro-rhDPPI by both enzymes was very similar to the processing by the v-cath peptidase and to the processing of the native enzyme (*22*, *24*).

Mainly on the basis of the results of pro-rhDPPI processing by cathepsin L, a plausible mechanism for DPPI maturation inside cells can be proposed. Zymogen dimers, formed immediately after biosynthesis, are transported to the endosomes/lysosomes by the mannose 6-phosphate receptor pathway, as shown in rat macrophages (*21*). Catalytic processing of the zymogen dimer inside lysosomes is initiated by cleavage of each monomer between the residual pro part and the activation peptide. This step is followed by cleavage in the C-terminus of the activation peptide, leaving a short extension on the heavy chain N-terminus. These two cleavages result in excision of the majority of the activation peptide. Finally, the extension on the heavy chain is (partially) excised by DPPI or a different peptidase, and the catalytic region is cleaved into the heavy and light chains by a single specific cleavage.

The stage at which the dimers associate to form tetramers is not known. According to the proposed function of the activation peptide in preventing assembly of the zymogen dimers, tetramers may form immediately after excision of either one or both of the activation peptides present in the zymogen dimers. On the other hand, recombinant rat DPPI was reported to exist as a dimer in the active form (*47*). This suggests that tetramer formation is not required for completion of the activation process and that the interaction between pairs of processed dimers is less strong than the interaction between the monomers that form the dimers.

The high activity, wide tissue distribution, and ability to process the zymogen point to cathepsin L as the primary activator of pro-DPPI in vivo. Despite a somewhat lower activation rate and a more restricted tissue distribution, cathepsin S and related endopeptidases may also participate in the processing in certain cell types and to a minor extent in general. Cathepsin B was unable to process the zymogen even under slightly acidic conditions, which generally favor the enzyme's endopeptidase activity over its carboxypeptidase activity (*55*), suggesting that only true endopeptidases are involved in DPPI activation. The idea that pro-DPPI is processed within the lysosomes by papain-like cysteine peptidases is supported by previous investigations. McGuire et al. (*1*) demonstrated that active DPPI is present inside lysosomes of human lymphocytes and myeloid cells, whereas Muno et al. (*21*) demonstrated the presence of DPPI zymogen and its active form in endosomes/lysosomes in rat macrophages. Maturation of rat pro-DPPI has also been studied in COS 7 cells transfected with pro-DPPI DNA (*56*). However, the results of this study are questionable, primarily due to the use of antibodies with insufficient specificity as discussed previously (*1*, *21*).

In contrast to the cysteine peptidases, the lysosomal aspartic peptidase cathepsin D degraded both the zymogen and the mature rhDPPI. The colocalization of hDPPI and cathepsin D within lysosomes and the high concentration of the latter (*57*) suggest a considerable intracellular turnover of hDPPI. Extracellular DPPI, however, can be regulated by cystatins, which efficiently inhibited rhDPPI in vitro. To perform extracellular functions, mature DPPI released by degranulation will therefore have to overcome the inhibitory potential of cystatin C (*37*). The biological significance of the inhibition of rhDPPI by stefins A and B depends on a possible colocalization of these proteins in vivo.

In conclusion, the first detailed characterization of the hDPPI zymogen and its activation by cysteine peptidases, in particular, human cathepsin L, have been presented. These results may promote future studies on the activation of pro-hDPPI in vivo and the possible impact of this process on the activation and activities of granule serine peptidases.

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