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The Cytoplasmic Peptidase DPP9 Is Rate-limiting for Degradation of Proline-containing Peptides*[S]

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Protein degradation is an essential process that continuously takes place in all living cells. Regulated degradation of most cellular proteins is initiated by proteasomes, which produce peptides of varying length. These peptides are rapidly cleaved to single amino acids by cytoplasmic peptidases. Proline-containing peptides pose a specific problem due to structural constrains imposed by the pyrrolidine ring that prevents most peptidases from cleavage. Here we show that DPP9, a poorly characterized cytoplasmic prolyl-peptidase, is rate-limiting for destruction of proline-containing substrates both in cell extracts and in intact cells. We identified the first natural substrate for DPP9, the $RU1_{34-42}$ antigenic peptide (VPYGSFKHV). $RU1_{34-42}$ is degraded *in vitro* by DPP9, and down-regulation of DPP9 in intact cells results in increased presentation of this antigen. Together our findings demonstrate an important role for DPP9 in peptide turnover and antigen presentation.

Protein turn-over is an essential process that continuously occurs in all living cells. The ubiquitin-proteasome system is responsible for initiating the regulated degradation of most cellular proteins (1). Proteasome-degradation products are not single amino acids but rather peptides varying in length between 3 and 22 amino acids (2, 3). Cytosolic amino- and endopeptidases rapidly cleave these peptides (4) to allow recycling of amino acids and to prevent accumulation of short peptides, which may be harmful to the cell. In addition, these peptidases also play an important role in the trimming of proteasomal products for antigen presentation on MHC[4] class I (5–8).

Peptides containing proline residues pose a problem for most peptidases due to the pyrrolidine ring of proline that gives it an exceptional conformational rigidity. Only few peptidases are known to cleave after prolines, including the cytoplasmic peptidases prolyl oligopeptidase (POP) and cytoplasmic members of the S9B/DPPIV family (DPP8 and DPP9). POP is a cytosolic endopeptidase of the S9A family, which is broadly distributed with high concentrations in the brain. It has been implicated in the maturation and degradation of peptide hormones and neuropeptides (9, 10).

S9B/DPPIV peptidases are a family of exopeptidases that cleave off N-terminal dipeptides from proteins/polypeptides having a proline residue at the second position (Xaa-Pro). The best-characterized member of this family is DPPIV, a membrane protein with a catalytic domain facing the extracellular space. DPPIV knock-out mice show enhanced insulin secretion and improved glucose tolerance (11, 12). This is due to cleavage and, thus, inactivation of the incretin hormones glucagon-like peptide and glucose-dependent insulinotropic polypeptide by DPPIV (13–15). Therefore, DPPIV is used as a drug target for the treatment of diabetes type 2.

In contrast, DPP8 and DPP9 are soluble cytoplasmic peptidases of unknown function. They share 60% amino acid identity and are ubiquitously expressed in vertebrate tissues (16–20). Because DPP8 and DPP9 knock-out mice are not available, most studies on these enzymes were done with inhibitors either against the DPPIV family or specifically against DPP8 and -9. Currently two specific DPP8/9 inhibitors are described (21, 22), of which one showed severe effects in animal models (21).

Here we show that DPP9 is a rate-limiting enzyme for cytosolic post-proline aminodipeptidase activity. Our work associates an *in vivo* function with DPP9 in peptide degradation and also suggests that changes in DPP9 expression levels or activity contribute to changes in the repertoire of cytosolic peptides, including those presented by MHC class I.

EXPERIMENTAL PROCEDURES

Antibodies—RU1 rabbit polyclonal antibody was produced as described (23). Mouse monoclonal antibodies against β-actin (clone AC-15) were purchased from Sigma. Anti-β-tubulin, POP, DPP8, and DPP9 antibodies were purchased from Abcam.

Plasmids—Full-length human cDNAs for DPP8 and DPP9 (IRATp970G0924D6 and IRALp962J0230) were obtained from the German Resource Center for Genome Research (RZPD). For bacterial expression, DPP8 and DPP9 were cloned

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[4] The abbreviations used are: MHC, major histocompatibility complex; POP, prolyl oligopeptidase; AMC, 7-amino-4-methylcoumarin; CTL, cytolytic T lymphocyte; siRNA, small interfering RNA; GFP, green fluorescent protein; ER, endoplasmic reticulum; IFN, interferon.

upstream of an intein/chitin binding domain in the pTXB3 vector (Biolabs) using the EcoRI and SapI sites.

Peptides—The fluorogenic substrates GP-7-amino-4-methylcoumarin (AMC), WP-AMC, VP-AMC, DP-AMC, AAF-AMC, succinyl-LLVY-AMC, and R-AMC were purchased from Biomol. MGP-AMC, KP-AMC, and GR-AMC were purchased from Bachem. These derivatives were more than 90% pure. All other peptides used in this study were purchased from Pepscan and are at least 75% pure. By mass spectroscopy analysis, no other major peptide species was detectable.

Inhibitors—The DPPIV-family inhibitor P32/98 was purchased from Biomol. The DPP8/9-specific inhibitor described as compound 2 in (21) was a kind gift from Jonathan Rosenblum, ActivX Biosciences. The proteasome inhibitor epoxomicin was purchased from Calbiochem.

Cell Culture and siRNA Experiments—HeLa and 293-EBNA cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. DPP8-1 siRNA (TGACGCCACTAATTATCTA), DPP9-1 siRNA (TGCACTTTCTACAGGAATA), DPP9-2 siRNA (GCCACCAAGGUUUAUCCAA), DPP9-3 siRNA (GGAUCAAUGUUCAUGACAU), and POP siRNA (CAGGTTTGCAGAACCAGCGAGTATT) were purchased from Invitrogen. For *in vitro* activity assays, HeLa cells in 24-well plates were transfected with 20 nM siRNA using Oligofectamine (Invitrogen) according to the manufacturer's instructions. 48 h after transfection, cells were split and retransfected. Cells were trypsinized 72 h after initial transfection, washed in phosphate-buffered saline, and resuspended in cold TB buffer (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 1 mM dithiothreitol). Cells were Dounce-homogenized and centrifuged at 100,000 × *g* for 60 min to remove membranous organelles. Protein content of the supernatant was measured, and 5 μg of protein lysates were tested for activity against 0.1 mM *X*P-AMC at 30 °C.

Recombinant Protein Purification—DPP8 or DPP9 in pTXB3 was transformed into *Escherichia coli* BL21 (Stratagene). Cells were grown to A_{600} 0.8 and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 16 h at 15 °C. Cells were harvested and resuspended in lysis buffer (20 mM HEPES, pH 8, 500 mM NaCl, 0.1 mM EDTA), supplemented with 0.1% Triton-X100, and disrupted by using an EmulsiFlex (Avestin). The 100,000 × *g* supernatant was passed over chitin beads (New England Biolabs) at 4 °C. Beads were washed at 4 °C with lysis buffer and then at room temperature with the same buffer supplemented with 10 mM $MgCl_2$ and 5 mM ATP. Proteins were eluted by incubation of the beads for 16 h at room temperature with 20 mM HEPES, pH 8, 500 mM NaCl, 0.1 mM EDTA, 50 mM dithiothreitol. Eluates were dialyzed against 20 mM HEPES, pH 8, 0.1 mM EDTA, 1 mM dithiothreitol and further purified using anion exchange chromatography (Mono Q, GE Healthcare).

In Vitro Activity Assay—50 nM purified DPP8/9 was incubated with 200 μM peptide (VPYGSFKHV). Reactions were carried out for 2 h in TB buffer at 30 °C in the presence or absence of 2.5 mM inhibitor (P32/98). Reactions were stopped by the addition of trichloroacetic acid and centrifugation at 4 °C (14,000 × *g* 10 min). The supernatant was diluted 80-fold and analyzed by matrix-assisted laser desorption ionization mass spectrometry. 0.5–1-μl samples were analyzed on a Bruker Reflex IV (Bruker Daltonics, Bremen, Germany) in reflectron mode using the thin-layer technique (24) for matrix preparation. Data processing was carried out using the XMass software (Bruker Daltonics).

For fluorimetric assays, 50 nM purified enzyme was incubated with 15, 31, 62.5, 125, 250, or 500 μM or 1 mM GP-AMC in a final reaction volume of 20 μl (Cliniplate 384-well plates, Labsystems) at 30 °C in TB buffer with 0.2% Tween and 0.2 mg/ml ovalbumin. Fluorescence was measured using a Fluoroskan Ascent microplate fluorimeter (Labsystems) with 380-nm (excitation) and 450-nm (emission) filters and Ascent software. K_m, V_{max}, k_{cat}, and K_i values were calculated using the Prism software. Each assay was repeated at least three times.

Competition Assays—Two peptide libraries were tested in competition assays with GP-AMC, *X*PYGSFKHV and VP*X*GSFKHV, where *X* is 1 of 20 amino acids, as well as tumor antigen peptides described under "Results." 50 nM recombinant enzyme was added to a premix of 100 μM GP-AMC and 400 μM test peptide, and emission at 450 nm was measured after 5 min and analyzed as described above. To determine the K_i of the tested peptides, different concentrations of GP-AMC (15, 31, 62.5, 125, 250, and 500 μM and 1 mM) were added to 0, 200, or 400 μM competing peptide, and GP-AMC hydrolysis was measured. Each assay was repeated at least three times.

Cytolytic T Lymphocyte (CTL) Activity Assays—For CTL activity assays, renal carcinoma cell line BB64-RCC (23) and 293-EBNA cells (Invitrogen) were grown in Iscove's medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone, Logan), arginine (116 mg/liter), glutamine (219 mg/liter), asparagine (36 mg/ml), and antibiotics. $RU1_{34-42}$ CTL clone 381/84 was isolated and grown as described (23). Cells plated on a 6-well plate were transfected with 40 nM DPP9-siRNAs or as control enhanced GFP-siRNA (EGFP-S1 DS, IDTDNA). 24 h later 293-EBNA cells were transiently transfected with RU1 and HLA-B51 encoding pcDNA3 plasmids (0.8 and 1.5 μg/well, respectively). 48 h after siRNA transfection, cells were harvested and plated in a 96-well plate (20–40,000 cells/well). For control, one half of the wells were loaded for 1 h before the CTL assay with the antigenic peptide (VPYGSFKHV, 1 μg/ml). Next, the CTL clone 381/84 was added (20,000 cells per well) in the presence of interleukin-2 (25 units/ml). The supernatants were collected 16 h later, and their interferon-γ content was measured by enzyme-linked immunosorbent assay (BIOSOURCE-Invitrogen). To evaluate the efficiency of DPP9 silencing, aliquots of BB64 and 293-EBNA transfectants were collected before the CTL assay. Cells were washed in cold phosphate-buffered saline, lysed in TB buffer, centrifuged at 20,000 × *g* for 20 min, and protein concentration was determined by BCA (Pierce).

Treatment of 293-EBNA Cells with DPP8/9 Inhibitor—293-EBNA cells growing in 6-well plates (2×10^6/well) were transfected with pCDNA3 plasmids coding for RU1 (0.8 μg/well), MAGE-A3 (0.8 μg/well), HLA-B51 (1.1 μg/well), and HLA-A1 (1.1 μg/well) using Lipofectamine. 16 h later MHC-bound peptides were eluted from the cells by incubation with a citrate buffer, pH 3, for 1.5 min at room temperature. Cells were then returned to fetal calf serum-free medium (X-vivo 10) at 37 °C. 2 h later cells were washed in phosphate-buffered saline and

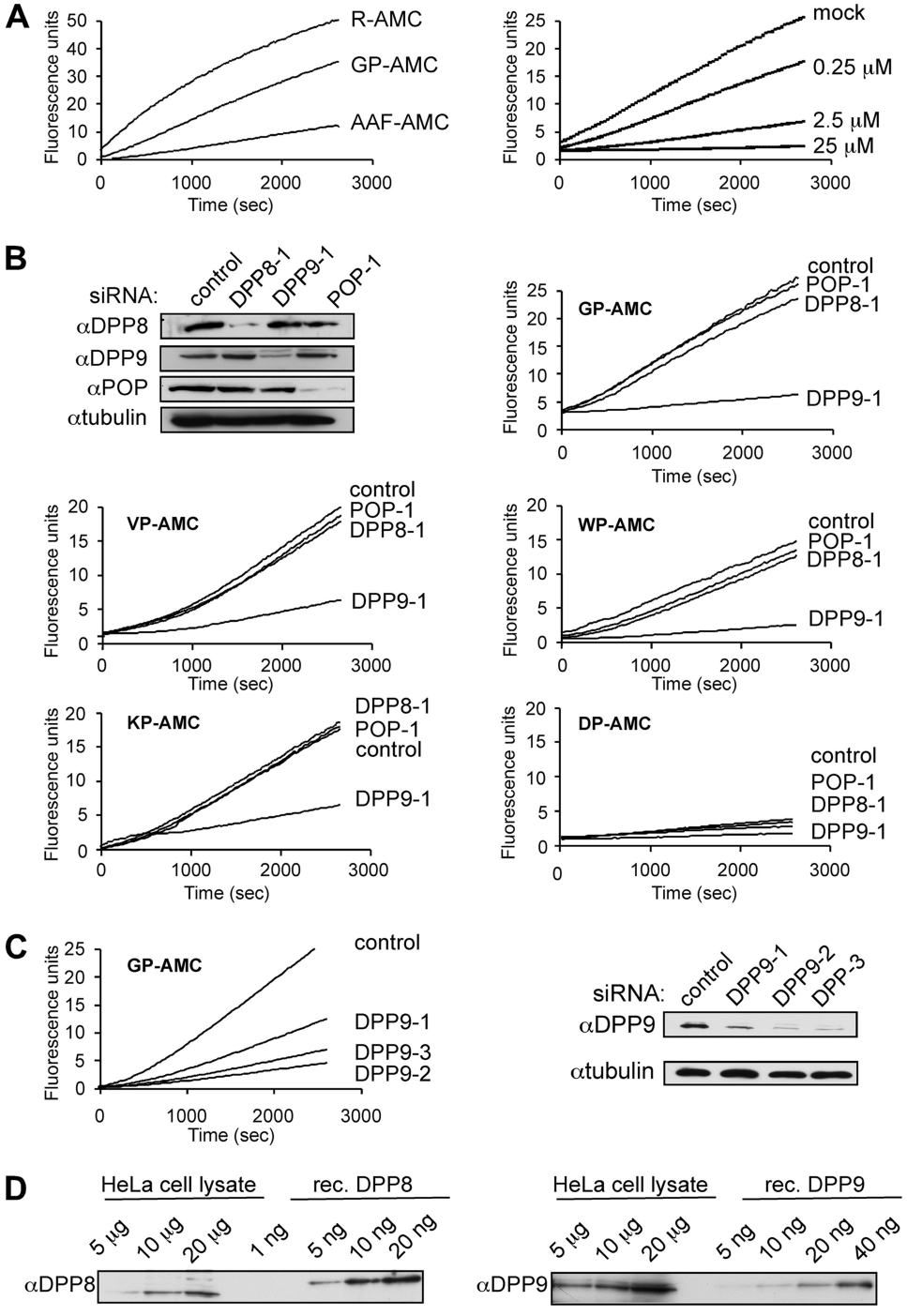
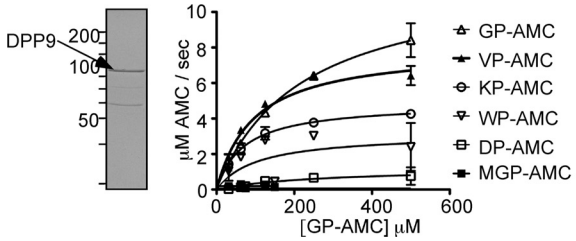


FIGURE 1. **A major fraction of cytosolic post proline peptidase activity is contributed by DPP9.** *A*, *left*, 5 μg of cytosol from HeLa cells were tested for the cleavage of R-AMC, GP-AMC, and AAF-AMC. Reactions were performed for the indicated times, and fluorescence was measured using 380-nm (excitation) and 450-nm (emission) filters. *Right*, 5 μg of HeLa cell cytosol were treated with the indicated concentrations of a specific DPP8/9 inhibitor (*compound 2*) before the addition of 250 μM GP-AMC. *B*, HeLa cells were transfected with siRNA against POP, DPP8, DPP9, or control siRNA and harvested after 72 h. Shown is a Western blot of cytosol extracts from the siRNA treated cells (10 μg of protein extract per lane) developed with antibodies against DPP8, DPP9, POP and tubulin as a loading control. Cytosol fractions from silenced cells were then tested for release of AMC from GP-AMC, KP-AMC, VP-AMC, WP-AMC, and DP-AMC as in *A* (5 μg of cytosolic fractions were used per reaction). *C*, cells were transfected with three different DPP9 siRNAs or control siRNA and analyzed for activity against GP-AMC as in *A*. A Western blot is shown to confirm the down-regulation of DPP9 in the analyzed fractions, developed with antibodies against DPP9 and tubulin. All kinetic assays (*A–C*) were repeated at least three times in triplicate; shown are results of one triplicate assay. *D*, to estimate DPP8 and DPP9 protein levels at steady state, three different concentrations of HeLa total cell lysates (5, 10, or 20 μg) and defined concentrations of recombinant (*rec*) DPP8 (1, 5, 10, 20 ng) or DPP9 (5, 10, 20, 40 ng) purified from *E. coli* were compared by immunoblotting with DPP8- or DPP9-specific antibodies.

resuspended in a low conductivity cytoporation buffer at 10^7 cells/ml. Cell suspensions (200 μl) were electroporated (200 V, 300 microfarads, 100 ohms) either with DMSO (mock treatment) or with 100 μM DPP8/9 inhibitor (stock at 20 mM in DMSO). Electroporated cells were incubated for 4 h in fetal calf serum-free medium (X-vivo 10). Mock-treated cells were then treated either with 0.5% DMSO (negative control), 1 μM epoxomicin, or 100 μM DPP8/9 inhibitor. Cells resuspended in CTL medium were split in two parts, and 5 μg/ml concentrations of antigenic peptides ($RU1_{34-42}$ or $MAGE\text{-}A3_{168-176}$) was added during 1 h. Cells washed in the CTL medium were used as targets for a CTL assay (40,000 targets for 20,000 CTLs) by the addition of the CTL clones 381/84 or A10. Each condition was tested in triplicate. To evaluate the efficiency of DPP8/9 inhibition, aliquots of the inhibitor-treated 293-EBNA cells were collected before the CTL assay. Cells were washed in cold phosphate-buffered saline, lysed in TB buffer, centrifuged at 20,000 × *g* for 20 min, and protein concentration was determined by BCA (Pierce). Cell extracts (5 μg) were tested for GP-AMC degradation as described above.

RESULTS

DPP9 Is a Rate-limiting Post-proline Aminodipeptidase in Cytosolic Extracts—Most cytosolic peptidases are inactive toward the amino acid proline because of the rigid geometric configuration of the prolyl-peptide bond. Nevertheless, the artificial substrate Gly-Pro-AMC (GP-AMC) is rapidly degraded in cytosolic extracts, as can be measured by the release of the fluorescent product AMC upon cleavage (Fig. 1*A*). Three post-proline peptidases are known to localize to the cytosol, POP, DPP8, and DPP9. Currently the function of DPP8 and DPP9 is not understood. We tested their contribution to the degradation of proline-containing peptides using a highly specific



	GP-AMC	KP-AMC	DP-AMC	WP-AMC	VP-AMC	MGP-AMC
k_{cat} (S^{-1})	121 ± 14	52,6 ± 1,4	54,0 ± 10	40,3 ± 2,6	79,9 ± 8	1,95 ± 0,5
K_m (μM)	222 ± 50	122 ± 10	> 1200	72,6 ± 16	96,0 ± 14	ND
k_{cat} / K_m ($M\ S^{-1}$)	$5*10^5$	$4,2*10^5$	ND	$5,5*10^5$	$8*10^5$	ND

FIGURE 2. ***In vitro* processing of *X*P-AMC substrates by recombinant DPP9.** *Left*, Coomassie staining of recombinant DPP9, expressed and purified from *E. coli*. The *graph* shows the results of a Michaelis-Menten assay performed as a triplicate assay using 50 nM DPP9 and varying concentrations of the *X*P-AMC substrate. The table shows calculated k_{cat}, K_m, and k_{cat}/K_m results for different substrates. Values were calculated using non-linear regression (Prism software).

DPP8/9 inhibitor, previously described as compound 2 by Lankas *et al.* (21). As shown in Fig. 1*A*, the addition of the DPP8/9 inhibitor to cytosolic extracts strongly inhibits GP-AMC degradation, suggesting that these peptidases and not POP are responsible for GP-AMC cleavage.

To differentiate between DPP8 and DPP9, HeLa cells were transfected for 72 h with DPP8 or DPP9 siRNA. For control, cells were transfected with siRNA against POP and a non-targeting siRNA. Cytosolic extracts of the silenced cells were then tested for GP-AMC cleavage. Depletion of POP or DPP8 had no significant effect on the cleavage of GP-AMC compared with treatment with control siRNA (Fig. 1*B*). On the other hand, depletion of DPP9 strongly reduced release of AMC from GP-AMC, suggesting that DPP9 is rate-limiting for cleavage of this peptide in HeLa cell lysates. Peptides that lack proline such as Gly-Arg-AMC, Arg-AMC, Ala-Ala-Phe-AMC, and *N*-succinyl-Leu-Leu-Val-Tyr-AMC were not stabilized in cells treated with siRNA against DPP8, DPP9, or POP (data not shown).

To test whether DPP9 is also rate-limiting for other proline-containing peptides, cytosolic extracts were tested for the degradation of Val-Pro-AMC (VP-AMC), Trp-Pro-AMC (WP-AMC), Lys-Pro-AMC (KP-AMC), and Asp-Pro-AMC (DP-AMC). Although Asp-Pro-AMC (DP-AMC) appeared to be very stable compared with the other *X*P-AMC substrates tested and was not affected by DPP9 silencing, all other *X*P-AMC substrates were significantly stabilized in DPP9- but not in DPP8 or POP-silenced cells (Fig. 1*B*).

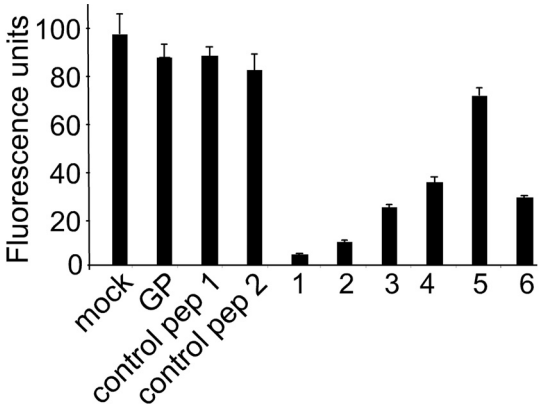
To ensure that the stabilizing effect of DPP9 silencing on *X*P-AMC substrates was not due to off-target effects, we repeated the experiment using two additional DPP9 siRNAs. Each of the three siRNAs led to strong reduction of post-proline cleavage activity in cell lysates, and the extent of reduction correlated well with the reduction in DPP9 levels (compare activity assays in Fig. 1*C* with the corresponding Western blot). Similar effects for DPP9 silencing were observed also in 293-EBNA cells (supplemental Fig. 2).

Next we compared the protein levels of DPP8 and DPP9 using recombinant enzymes for normalization of the antibody. In several different cell lines DPP9 is a very abundant protein accounting for up to 0.4% of total cellular proteins (Fig. 1*D* and supplemental Fig. 3). In contrast, full-length DPP8 is barely detected in cellular lysates. A slightly shorter band which disappears under siRNA treatment can be detected in most cell lines tested. Whether this band represents a splice variant or a limited proteolysis product of DPP8 is currently unknown. However, its levels are still ~10-fold lower than those of DPP9.

Next we verified that DPP9 cleaves GP-AMC, KP-AMC, WP-AMC, VP-AMC, and DP-AMC using *in vitro* assays with recombinant enzyme, which was expressed and purified from *E. coli* (Fig. 2). In accordance with the relatively high stability of DP-AMC in cell lysates, we find that it is the least efficient substrate for recombinant DPP9. Peptides that did not contain a proline residue were not degraded by DPP9 (not shown). Taken together, these findings suggest that at least 80% of the cytosolic post-proline cleavage activity measured on the artificial substrates GP-AMC, KP-AMC, VP-AMC, and WP-AMC can be attributed to DPP9.

Proline-containing Antigen-putative Substrates for DPP9—So far no physiological or natural substrates of DPP8 or DPP9 have been identified. However, our data suggest that DPP9 is necessary for cleavage of many different proline-containing peptides, implying a housekeeping function for DPP9 in peptide turnover. If this is the case, DPP9 may also influence the pool of antigenic peptides that are loaded on MHC class I, as some of the peptides that emerge from the proteasome are used for antigen presentation. For binding to MHC class I, peptides typically require a length of 8–10 amino acids. Because proteasomal products are often longer (2, 3), they are trimmed on the N terminus either after translocation into the ER (25–28) or in the cytoplasm by amino- and endopeptidases (29–35). Antigen peptides with a proline residue in second position are frequently found in association with HLA-B7, HLA-B8 HLA-B15, HLA-B51, HLA-B35, and HLA-B53 (36). Hence, such antigens are putative *in vivo* substrates for DPP8 and DPP9.

We selected several known peptide antigens that contain an anchor proline residue in position two (www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm) and tested them in competition assays with GP-AMC. Michaelis-Menten analysis shows that each of these peptides acts as a competitive inhibitor, albeit with different efficiency (Fig. 3 and data not shown). Tumor-associated antigens from mutated CASP-8 (^{476}FPSDSWCYF^{484}) and gp100 (^{471}VPLDCVLYRY^{480}) are very efficient competitors, and gp100 (^{630}LPHSSSHWL^{638}) and LAGE-2 (^{94}MPFATPMEA^{102}) compete well, whereas the LAGE-1 antigen (^{46}APRGVRMAV^{54}) is a poor competitor (K_i values varied between 3.6 and 381 μM). For control we used proline-free peptides, which did not inhibit AMC release from GP-AMC (control peptides *1* and *2*, Fig. 3). The dipeptide GP, which is the product of GP-AMC cleavage, did not cause product inhibition. Based on these findings, DPP9 appears to be able to cleave a wide range of naturally occurring peptide antigens



Protein	Peptide epitope	Ki μM
1. CASP-8	FPSDSWCYF	3,6 ± 0,9
2. gp100	VPLDCVLYRY	5 ± 0,7
3. gp100	LPHSSSHWL	79,8 ± 25,4
4. LAGE-2	MPFATPMEA	122,4 ± 20,8
5. LAGE-1	APRGVRMAV	381.8 ± 40,1
6. RU1	**VPYGSFKHV**	**30 ± 2,2**

FIGURE 3. **Antigens containing proline at position 2 are competitive substrates for DPP9.** GP-AMC (100 μM) was mixed with a competing peptide (400 μM) before the addition of 50 nM recombinant DPP9. AMC release was measured after 5 min using 380 nm (excitation) and 450 nm (emission) filters. Sequence of control peptides 1 and 2 are EQKLISEEDL and DYKDDDDK, respectively. K_i values were calculated using detailed Michaelis-Menten analysis in competition assays with GP-AMC (by mixing 0, 100, or 500 μM peptide with varying concentrations of GP-AMC) using the Prism software. Each experiment was repeated at least four times (each in triplicate); shown are the results of one experiment done in triplicate.

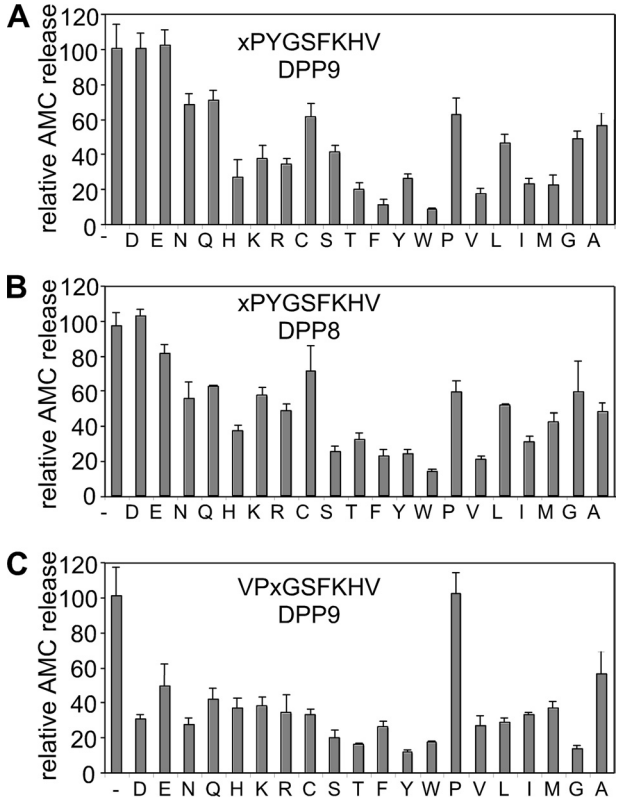


FIGURE 4. **Recombinant DPP8 and DPP9 share similar substrate specificity.** Two peptide libraries were tested in competition assays with GP-AMC, *X*PYGSFKHV and VP*X*GSFKHV, where *X* is one of 20 amino acids. GP-AMC (100 μM) was mixed with a competing peptide (400 μM) before the addition of 50 nM recombinant DPP8 or DPP9. AMC release was calculated 5 min after initiation of reaction. Relative AMC release was calculated by setting AMC release in the absence of competing peptide to 100%. *A*, competition assays for DPP9 with peptide library *X*PYGSFKHV. *B*, competition assays for DPP8 with peptide library *X*PYGSFKHV. *C*, competition assays for DPP9 with peptide library VP*X*GSFKHV. Each experiment was performed at least four times, each in triplicate. Shown are the results of a triplicate.

with proline in second position but shows significant substrate preference.

DPP8 and DPP9 Display Similar Substrate Specificity—Next we asked whether DPP8 and DPP9, which that are 60% identical, have overlapping substrate specificity. To get insights into parameters that determine efficient cleavage by DPP8 and DPP9, we carried out a detailed competition analysis with peptides that vary only in one amino acid. As described above, selected antigens competed with GP-AMC for cleavage by DPP9, but they varied significantly in efficiency. As a model substrate we chose the $RU1_{34-42}$ antigen (VPYGSFKHV) as it had the potential to be a physiological substrate (see below). First, we confirmed that the VPYGSFKHV peptide is indeed cleaved by recombinant DPP8 and DPP9. Mass spectrometry analysis revealed that both enzymes remove the first two amino acids from the VPYGSFKHV peptide (supplemental Fig. 4, *A*, *B*, and *D*). Proteolytic cleavage was blocked by isoleucine-thiazolidide (P32/98), a specific competitive inhibitor for peptidases of the DPPIV family, including DPP8 and DPP9 (supplemental Fig. 3, *C* and *E*) (21). We then tested 20 variants of the $RU1_{34-42}$ peptide, differing only in the first amino acid, in competition studies (Fig. 4, *A* (for DPP9) and *B* (for DPP8)). Michaelis-Menten analysis demonstrated that the peptides act as competitive inhibitors of GP-AMC (data not shown). Based on these data, DPP8 and DPP9 showed very similar catalytic preferences. Both prefer substrates with an aromatic (Phe, Tyr, Trp) or branched aliphatic (Val, Ile) amino acids at the first position. Poor competitors are acidic residues (Glu and Asp), which is in line with our observation of the high K_m for the degradation of DP-AMC by DPP9. Next we tested whether DPP9 has preferences for the amino acid after proline by using variants of the RU1 peptide that differ in the third amino acid (P2). The only peptide that was a poor competitor was VPPGSFKHV, suggesting that DPP9 cannot cleave a Pro-Pro bond (Fig. 4*C*).

DPP9 Restricts MHC Class I Presentation of a Proline-containing Antigen—For most antigens it is unknown whether they are generated in the cytoplasm, where they could encounter DPP8 and DPP9, or whether they are matured in the ER. An exception is $RU1_{34-42}$ (VPYGSFKHV) (23), an antigenic peptide that was shown to be produced in the cytoplasm (37). $RU1_{34-42}$ was discovered as an antigen presented by MHC class I to CTLs raised against a renal carcinoma cell line (23).

Our competition assays in Figs. 3 and 4 suggest that the $RU1_{34-42}$ antigenic peptide VPYGSFKHV is an efficient substrate for DPP8 and DPP9 *in vitro*. Because DPP8, DPP9, and the $RU1_{34-42}$ peptide are present in the cytoplasm (16–18, 37), we asked whether $RU1_{34-42}$ is an *in vivo* substrate for both enzymes. Production and stability of the $RU1_{34-42}$ antigen can be assessed indirectly due to its presentation on the cell surface by MHC class I allele HLA-B51 to a cytolytic T lymphocyte

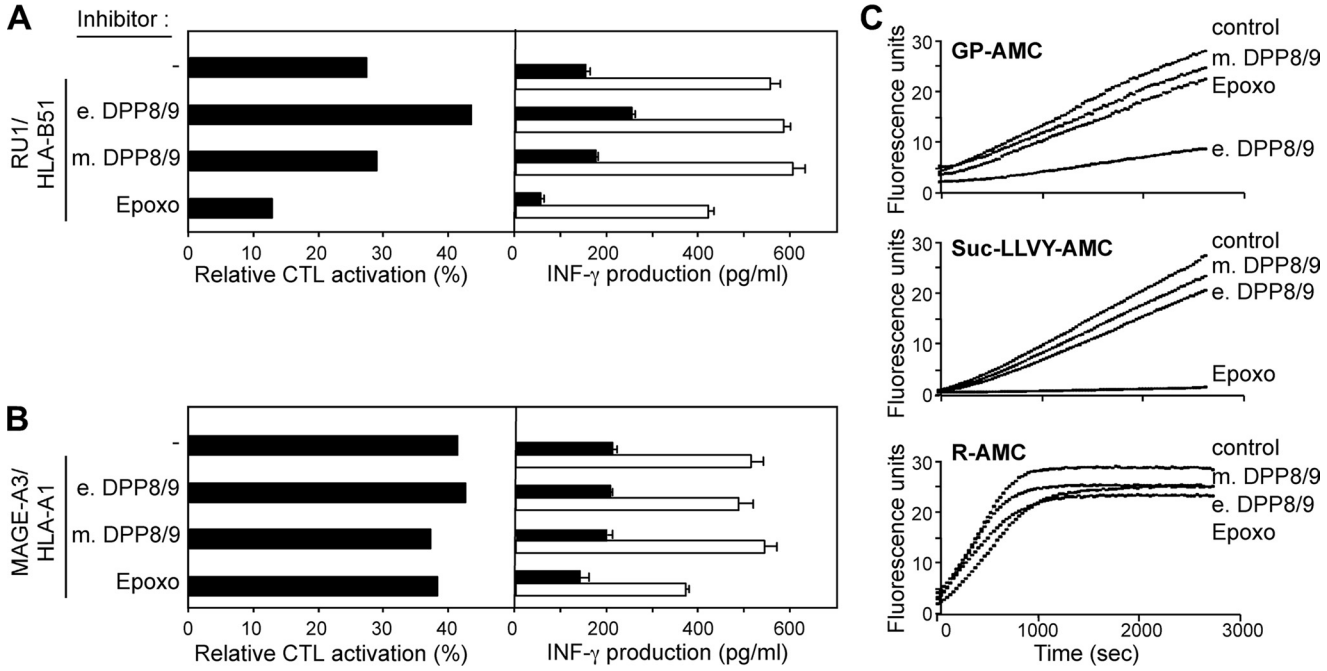


FIGURE 5. **Administration of a DPP8/9-specific inhibitor to cells leads to increased presentation of the $RU1_{34-42}$ antigen.** *A*, 293-EBNA cells were transiently transfected with plasmids expressing RU1 and HLA-B51. 48 h later cells were treated for 2 h with either 1 μM concentrations of the proteasome inhibitor epoxomicin (*Epoxo*) or 100 μM DPP8/9 specific inhibitor (*m. DPP8/9*), which was added to the medium. Alternatively, cells were electroporated with 100 μM DPP8/9 inhibitor (*e. DPP8/9*) or DMSO for control. Two hours later CTL clone 381/84 was added to the cells, and INF-γ release was measured (*black bars*, *right graph*). Saturating amounts of the antigenic peptide were added to cells before incubation with CTL clone 381/84 (*white bars*, *right graph*) to assess their viability. Shown on the left side is a simplified presentation of the inhibitor effect on the peptide presentation. It consists of the ratio between INF-γ release from tested cells (*black bars*, *right graph*) divided by INF-γ release from the control cells that were incubated with saturating amounts of the peptide before the assay (*white bars*, *right graph*). Each experiment was repeated at least three times, each time in triplicate (shown is one experiment in triplicate). The *error bars* show S.D. of the triplicates. *B*, cells in *A* were tested for their capacity to present $MAGE\text{-}A3_{168-176}$ to HLA-A1 using CTL clone A10. CTL clone A10 was isolated from a HLA-A1 melanoma patient vaccinated with a recombinant pox virus encoding MAGE-A3168–176 *C*, lysates of cells in *A* (5 μg) were tested for activity against 5 μM GP-AMC, 50 μM succinyl-LLVY-AMC, and 5 μM R-AMC. Each assay was repeated three times.

(CTL clone 381/84)(23). The CTL recognition can be monitored by measuring the interferon-γ produced by the activated CTLs. If DPP8 and/or DPP9 efficiently cleave the VPYGSFKHV peptide *in vivo*, then reduced levels or activity of these peptidases should lead to increased presentation of the $RU1_{34-42}$ antigen on the cell surface.

To test this hypothesis, we analyzed the effect of the DPP8/9-specific inhibitor (compound 2 (21)) on the presentation of the $RU1_{34-42}$ antigen to T-cells. To confirm inhibitor uptake, the degradation of GP-AMC was measured in extracts of treated cells. Surprisingly, we found that incubation of cells with 100 μM DPP8/9 inhibitor for 2 h did not affect the degradation of GP-AMC, suggesting that the inhibitor may not have entered the cells. On the other hand, cells electroporated with 100 μM DPP8/9 inhibitor showed a strong reduction in the degradation of GP-AMC in comparison to control cells that were electroporated with DMSO only (Fig. 5*C*). We then tested the capacity of the inhibitor-treated cells to present the $RU1_{34-42}$ antigen. As shown in Fig. 5, cells that showed reduced GP-AMC degradation (DPP8/9 inhibitor was electroporated) were more potent in presentation of the $RU1_{34-42}$ antigen (Fig. 5, *A* and *C*). This suggests increased stability of the $RU1_{34-42}$ antigen (VPYGSFKHV) due to inhibition of DPP8 and -9. We controlled the antigen production process by adding a specific proteasome inhibitor, epoxomicin, to the cells, as the 26 S proteasome is necessary for producing the C terminus of the $RU1_{34-42}$ antigen (23). Accordingly, treatment of cells with this inhibitor led to reduced presentation of the $RU1_{34-42}$ antigen and increased the stability of the proteasome substrate *N*-succinyl-Leu-Leu-Val-Tyr-AMC in cell lysates (Fig. 5, *A* and *C*). To control the specificity of the inhibitors, we followed the presentation of a tumor antigen $MAGE\text{-}A3_{168-176}$ that does not require the 26 S proteasome or serine peptidases for its presentation (Parmentier N).[5] As expected, $MAGE\text{-}A3_{168-176}$ presentation was unaffected by either inhibitor (Fig. 5, *B* and *C*). These results suggest that the $RU1_{34-42}$ antigen is an *in vivo* substrate of DPP8 and DPP9.

To differentiate between both enzymes we transfected DPP8 or DPP9 siRNA into the renal carcinoma cell line BB64-RCC in which the RU1 antigen was originally identified (23). Two days later, when DPP8 and DPP9 levels were efficiently down-regulated (Fig. 6, *A* and *B*), cells were tested for recognition by CTL clone 381/84. As shown in Fig. 6*B*, silencing of DPP8 did not have an effect on the presentation of the peptide compared with control cells transfected with a siRNA specific for enhanced GFP. However, we observed that interferon-γ production was two times higher with DPP9-silenced cells compared with the control cells (Fig. 6*A*). These results were confirmed using a second cell line, 293-EBNA cells, and three different DPP9-specific siRNAs. Because 293-EBNA cells do not express RU1 or HLA-B51, the expression of

[5] N. Parmentier and B. J. Van den Eynde, manuscript in preparation.

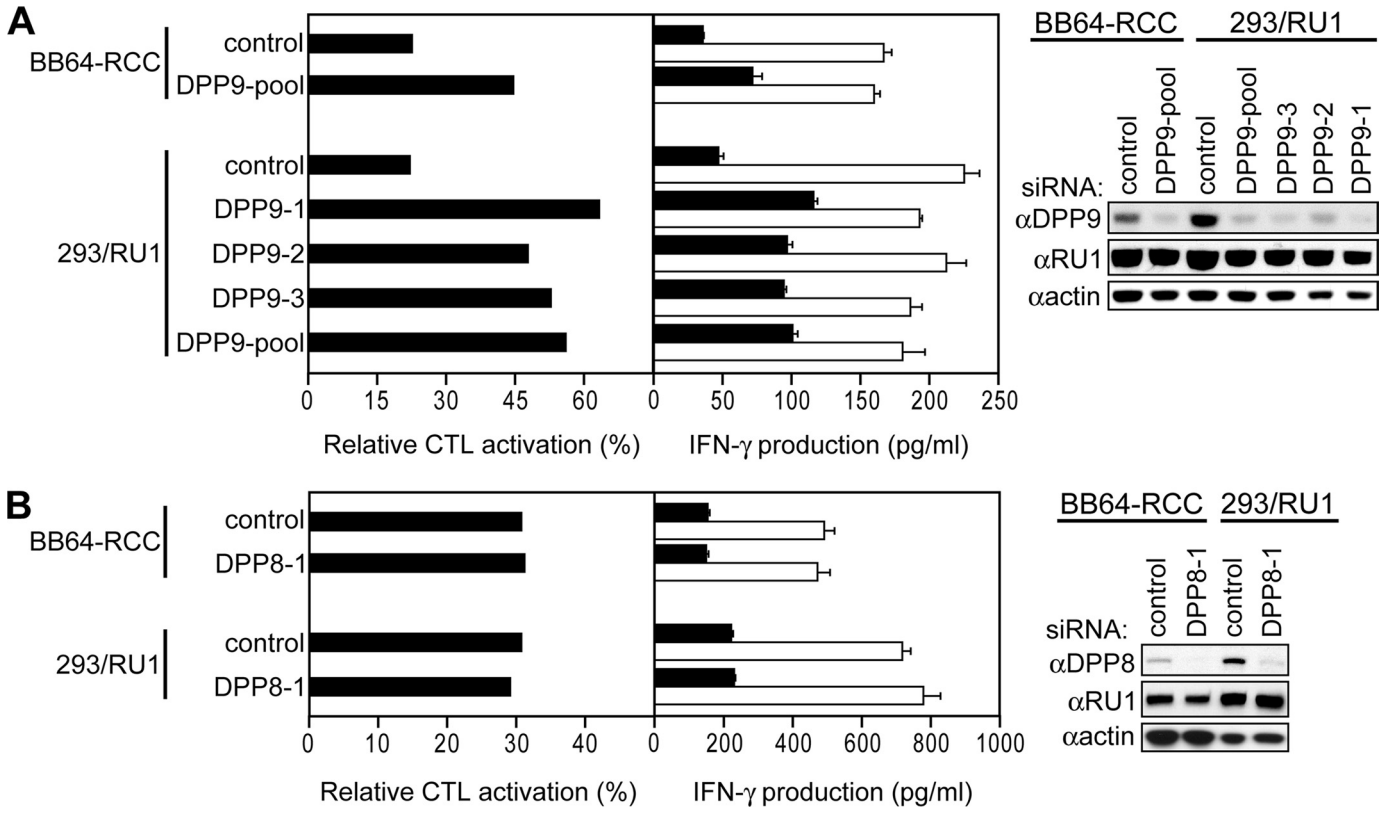


FIGURE 6. **The $RU1_{34-42}$ antigen is a substrate for DPP9 *in vivo*.** *A*, BB64-RCC and 293-EBNA cells were transfected with siRNA against DPP9 or against GFP as control. 24 h later 293-EBNA cells were transiently transfected with plasmids expressing RU1 and HLA-B51 (BB64-RCC express RU1 and HLAB51 endogenously). CTL clone 381/84 was added to the cells, and INF-γ release was measured (*black bars*, *right graph*). As a control, saturating amounts of the antigenic peptide were added to cells before incubation with CTL clone 381/84 (*white bars*, *right graph*) to assess the viability after siRNA treatment. Shown on the *left side* is a simplified presentation of the siRNA effect on the peptide presentation. It consists of the ratio between INF-γ release from tested cells (*black bars*, *right graph*) divided by INF-γ release from the control cells that were incubated with the peptide before the assay (*white bars*, *right graph*). Each experiment was repeated at least three times, each time in triplicate (shown is one experiment in triplicate). The *error bars* show S.D. of the triplicates. Shown is a Western blot analysis of the cells used as targets in the CTL assay. β-Actin was used as a loading control. *B*, same experiment as in *A*, except that cells were transfected with siRNA against DPP8 or GFP for control.

$RU1_{34-42}$ was induced using plasmids encoding both proteins. Again, in contrast to DPP8, DPP9 silencing resulted in increased interferon-γ production by the CTLs compared with cells transfected with control siRNA (*p* values were lower than 0.001). Silenced cells and control cells incubated with saturating amounts of the RU1 peptide were equivalently recognized by clone 381/84, indicating that DPP8/9-silencing did not affect cell viability (Fig. 6, *right panel*, *white bars*). The increase in RU1 presentation was not due to higher levels of the RU1 protein in DPP9-silenced cells (Fig. 6*A*). These observations let us to conclude that the $RU1_{34-42}$ antigen is a natural substrate for DPP9 and that endogenous DPP9 limits the presentation of this antigenic peptide.

DISCUSSION

DPP9 Accounts for Most Post-proline Dipeptidase Activity in the Cytoplasm—Most cytoplasmic peptidases cannot cleave after the amino acid proline, with the exception of POP, DPP8, and DPP9. Here we show that DPP9 is responsible for most cytosolic post-proline peptidase activity, at least on selected targets. Administration of a DPP8/9-specific inhibitor to HeLa and 293-EBNA cells strongly reduces the degradation of GP-AMC. In addition, depletion of DPP9, but not of DPP8 or POP, from HeLa and 293-EBNA cell extracts decreases the cleavage rates of several *X*P-AMC substrates. Finally, administration of a DPP8/9 inhibitor or siRNA treatment against DPP9 but not DPP8 protects the $RU1_{34-42}$ antigen from degradation both in BB64RCC as well as 293-EBNA cells. Hence, although recombinant DPP8 and DPP9 have very similar substrate specificity *in vitro*, their relative contributions both in extracts and in intact cells are very different. Consistent with this, DPP9 is much more abundant than DPP8 in several different cell lines including HeLa, 293-EBNA, and BB64RCC cells (supplemental Fig. 3 and data not shown). Taken together our data suggest a correlation between protein levels and enzymatic contribution of DPP8 and DPP9. With respect to development of specific inhibitors, it will be important to compare protein levels of DPP8 and DPP9 in different tissues but also throughout development and during stress conditions.

Substrate Specificity of DPP8 and DPP9—As revealed by competition assays, DPP8 and DPP9 have very similar substrate specificity. A key determinant for efficient cleavage by DPP8 and DPP9 is the first amino acid. Substrates with an aromatic or branched aliphatic amino acid at the first position are clearly preferred, whereas acidic residues are disfavored. These results are in agreement with a recent publication that determined the degradation of peptide libraries by DPP8 and DPP9 expressed

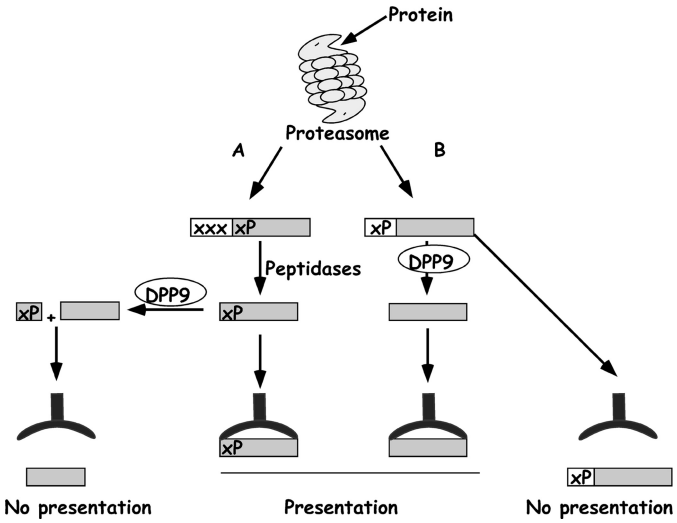


FIGURE 7. **DPP9 is a rate-limiting enzyme that contributes both positively and negatively to the repertoire of peptides presented by MHC class I.** Ubiquitinated proteins are degraded by the proteasome to peptides of varying length. N-terminal-extended versions of peptide antigens are among the many different peptides produced by proteasomal degradation (antigenic peptides are presented as *gray boxes*, and N-terminal extensions are presented as *white boxes*). These extensions are subsequently removed by endo- and aminopeptidases to produce the mature epitope. *A*, antigenic peptides such as RU1 contain a XaaPro at the N terminus and can be destroyed by DPP9 if they are present in the cytosol. In this case silencing of DPP9 results in increased presentation of the mature epitope. *B*, other antigenic peptides may depend on DPP9 for presentation if a XaaPro dipeptide flanks the N terminus of the mature antigen.

and purified from HEK293T cells (38). With the exception of proline, any amino acid is tolerated at the third position. Additional parameters such as length and/or C-terminal residues also seem to contribute to recognition and cleavage efficiency; although the gp100 antigen VPLDCVLYRY shares the first amino acid with the $RU1_{34-42}$ peptide, it is a much better competitor for GP-AMP (5-fold lower K_i). Full understanding of substrate preferences may require extensive analysis of hundreds of peptides or cocrystallization of DPP9 with model substrates.

A Role for DPP9 in Antigen Presentation—Antigenic peptides with proline in P2 are frequently found in association with HLA-B7, HLA-B8 HLA-B15, HLA-B51, HLA-B35, and HLA-B53 (36). However, such peptides are not efficiently transported through transporter associated with antigen processing into the ER unless proline is compensated for by additional favorable amino acids (39–42). It is, therefore, assumed that most antigens with proline in P2 are transported as N-terminal-extended precursors into the ER where they are further processed by ERAP1/ERAAP and ERAP2 (26, 27, 43). Although the site of maturation is unknown for most antigens, $RU1_{34-42}$ maturation was shown to take place in the cytoplasm. The final $RU1_{34-42}$ peptide (VPYGSFKHV) can then enter the ER where it associates with MHC class I molecules (37). In line with these results, we find that silencing or inhibition of the cytoplasmic peptidase DPP9 results in increased presentation of the $RU1_{34-42}$ peptide. Our data suggest that modulation in the expression levels or activity of DPP9 can determine whether the $RU1_{34-42}$ peptide is destroyed or presented at the cell surface and strengthen previous findings that proline-containing antigens can be matured in the cytoplasm and then transported into the ER. It will be interesting to see whether the $RU1_{34-42}$ antigen is an exception or whether many proline-containing antigens mature in the cytoplasm and are, thus, putative substrates for DPP9.

In conclusion, our work assigns a biological function to DPP9 as a rate-limiting enzyme for degradation of cytosolic proline-containing peptides. We have characterized the first endogenous substrate for DPP9 and added the enzyme to the short list of peptidases implicated in antigen presentation. From these findings we predict that DPP9 contributes both positively and negatively to the repertoire of peptides that is available for MHC class I presentation; antigens containing Xaa-Pro, such as $RU1_{34-42}$, could be destroyed by DPP9 (Fig. 7, *peptide A*), whereas antigen precursors with an N-terminal Xaa-Pro dipeptide may need DPP9 for their maturation (Fig. 7, *peptide B*). Whether DPP8 functions in a more specialized pathway or whether it plays a similar role to DPP9 is still an open question. Future work will be aimed at investigating whether changes in the activity of DPP8 and DPP9 due to physiological regulation pathophysiological changes, or drug treatment do indeed contribute to significant changes in the peptide repertoire presented by MHC class I. If so, DPP8 and DPP9 may be useful drug targets in immune-related disorders (discussed in Refs. 10 and 44). Application of the DPP8/9 inhibitor (compound 2) used in this study was previously reported to attenuate T-cell activation (21). However, experiments done by us and others (22) using the same inhibitor suggest that this inhibitor is not (easily) cell-permeable. Therefore, development of DPP8- and DPP9-specific cell-permeable inhibitors would be very important tools to further study the contribution of these two enzymes in cells and animal models.

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