Down-regulation of T Cell Activation following Inhibition of Dipeptidyl Peptidase IV/CD26 by the N-terminal Part of the Thromboxane A2 Receptor

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Using synthetic inhibitors, it has been shown that the ectopeptidase dipeptidyl peptidase IV (DP IV) (CD26) plays an important role in the activation and proliferation of T lymphocytes. The human immunodeficiency virus-1 Tat protein, as well as the N-terminal nonapeptide Tat(1–9) and other peptides containing the N-terminal sequence XXP, also inhibit DP IV and therefore T cell activation. Studying the effect of amino acid exchanges in the N-terminal three positions of the Tat(1–9) sequence, we found that tryptophan in position 2 strongly improves DP IV inhibition. NMR spectroscopy and molecular modeling show that the effect of Trp² Tat(1–9) could not be explained by significant alterations in the backbone structure and suggest that tryptophan enters favorable interactions with DP IV. Data base searches revealed the thromboxane A2 receptor (TXA2-R) as a membrane protein extracellularly exposing N-terminal MWP. TXA2-R is expressed within the immune system on antigen-presenting cells, namely monocytes. The N-terminal nonapeptide of TXA2-R, TXA2-R(1–9), inhibits DP IV and DNA synthesis and IL-2 production of tetanus toxoid-stimulated peripheral blood mononuclear cells. Moreover, TXA2-R(1–9) induces the production of the immunosuppressive cytokine transforming growth factor-β1. These data suggest that the N-terminal part of TXA2-R is an endogenous inhibitory ligand of DP IV and may modulate T cell activation via DP IV/CD26 inhibition.

Dipeptidyl peptidase IV (DP IV) (EC 3.4.14.5; Swiss-Prot accession number P27487) is an exopeptidase removing N-terminal dipeptides from oligopeptides with protonated N terminus if the penultimate amino acid is proline or alanine (1, 2). DP IV is a type II membrane glycoprotein and is identical to the activation antigen CD26 expressed on T lymphocytes, activated B lymphocytes, and natural killer cells. It has been shown that DP IV plays a key role in the regulation of differentiation and growth of lymphocytes (3). Specific DP IV inhibitors, such as Lys[Z(NO₂)]-thiazolidide and Lys[Z(NO₂)]-pyrrolidide, suppress mitogen- and alloantigen-induced T cell proliferation, B cell differentiation, and immunoglobulin secretion (4). Moreover, these DP IV inhibitors reduce the production of the cytokines IL-2, IL-10, IL-12, and interferon-γ and enhance that of the immunosuppressive cytokine TGF-β1. TGF-β1 is shown to be at least in part responsible for the immunosuppressive effects of DP IV inhibitors (5).

Peptides containing the N-terminal amino acid sequence XXP inhibit DP IV and exhibit similar suppressive effects on the activation of immune cells, as observed by using synthetic inhibitors (6–8). The human immunodeficiency virus-1 (HIV-1) transactivator Tat containing this N-terminal XXP motif is actively released by infected cells (9, 10) and exerts many pathological activities, including immunosuppressive effects on non-HIV-1-infected T cells (11). Indeed, natural Tat(1–86) inhibits DP IV (12, 13). Moreover, Tat(1–86) distinctly suppresses DNA synthesis of antigen- as well as mitogen-stimulated peripheral blood mononuclear cells (PBMNC) and T cells in the same concentration range as the highly specific DP IV inhibitor Lys[Z(NO₂)]-thiazolidide (IC₅₀ = 2.7 μM) (7, 8). This suggests that immunosuppressive effects of HIV-1 are at least in part mediated by Tat-DP IV interactions. The N-terminal nonapeptide Tat(1–9) (MDPVDPNIE) inhibits DP IV activity and DNA synthesis in a comparable extent to Tat(1–86), if it is used in a 20-fold higher concentration (7). Recently, we demonstrated the striking dependence of both DP IV inhibition and suppression of DNA synthesis, by Tat(1–9) and two Tat(1–9) analogues, Ile⁵-Tat(1–9) and Leu⁵-Tat(1–9), from their solution conformations. This provides further evidence for the mediation of antiproliferative effects of HIV-1 Tat via specific interactions of Tat with T cell-expressed DP IV (8). The function of the viral protein Tat as an immunomodulatory oligopeptide implies the existence of soluble or cell surface-expressed endogenous counterparts, e.g., on antigen-presenting cells, down-regulating the activation of T cells by inhibiting DP IV.

In this study, our strategy was to find peptidergic DP IV inhibitors by testing Tat(1–9) analogue peptides carrying exchanges in the first three amino acids. On the basis of the obtained N-terminal sequence, protein data base searches for potential ligands were performed. Of all examined Tat(1–9)-derived peptides, Trp²-Tat(1–9) turned out to be the most po...
tent DP IV inhibitor. A data base search for the N-terminal motif MWP of Trp<sup>2</sup>-Tat(1–9) discloses the thromboxane-A2 receptor (TXA2-R) (Swiss-Prot accession number P21731) sequence. The N-terminal nonapeptide of TXA2-R, TXA2-R(1–9), exerts inhibitory effects comparable to those of Trp<sup>2</sup>-Tat(1–9) on DP IV activity and on DNA synthesis of activated PBMC. Interestingly, TXA2-R is localized on the surface of monocytes known as antigen-presenting cells and intensively interacting with T cells during antigen presentation (14, 15). Therefore, TXA2-R on monocytes could represent a physiological inhibitor of T cell-expressed DP IV/CD26 and could be involved in immunomodulatory processes mediated via inhibition of DP IV enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Purification of Human Kidney DP IV and Soluble Recombinant Human DP IV**

DP IV was purified from human kidney cortex. Membrane proteins were solubilized by the addition of 1% Triton X-100 to the homogenized tissue. Subsequently, a fraction of ammonium sulphate precipitation was performed. The fraction between 50 and 65% saturation, containing most of the DP IV, was used for further purification by means of three different steps of liquid chromatography. After gel chromatography on Sepharose 6B, the pooled DP IV<sup>+</sup> fractions were applied on a Sephadex A50 ion exchange column and eluted with an increasing NaCl gradient. The final polishing step was performed with a FPLC POROS HQ ion exchange column. The resulting DP IV preparation has a specific activity of 32 units/mg and shows no contaminations upon silver-stained polyacrylamide gel electrophoresis.

Soluble human DP IV was produced recombinantly in Chinese hamster ovary cells (16). The cell culture supernatant of these transfected cells (gift from Dr. M. Gorrell, Sydney, Australia) was applied on a FPLC POROS HQ ion exchange column. The column was eluted with an increasing NaCl gradient. DP IV-containing fractions were analyzed by polyacrylamide gel electrophoresis (silver-stained) and the fractions without contaminations were pooled for further use.

**DP IV Substrates and Peptides**

The chromogenic DP IV substrate Ala-Pro-pNA was synthesized according to standard procedures (17). The substrate IL-2(1–12) and effector peptides were obtained by solid-phase peptide synthesis with Fmoc (N-(9-fluorenylmethyl)oxycarbonyl) technique using peptide synthesizer 431A (Applied Biosystems). The synthetic peptides were purified by reversed-phase HPLC and analyzed by mass spectrometry and elemental analysis.

**DP IV-catalyzed Hydrolysis of IL-2(1–12)**

Human kidney DP IV assay solution was prepared in 10 mM sodium phosphate buffer, pH 7.5, containing 2 mM EGTA to inhibit activities of putative metalloprotease impurities. After precubination (30 min at 37 °C) of 550 femtomolals of DP IV with 400 μM (final concentration in 10 mM sodium phosphate assay buffer, pH 7.5) of the effector peptide in Sigmoid-octated (Sigma) reaction vials, the enzymatic reaction was started by addition of IL-2(1–12) substrate (final concentration, 400 μM). Samples were incubated for 30 min at 37 °C. The reaction was stopped by addition of 30 mM phosphoric acid. Degradation of IL-2(1–12) was measured by capillary electrophoresis using Biofocus 3000 system (Bio-Rad). Separations were performed with a polyacrylamide-coated silica capillary (Bio-Rad; inner diameter 50 μm, length 30 cm) working at a constant voltage of 17 kV (positive to negative). For quantification of IL-2(1–12) His as internal standard was added (18).

**Determination of Inhibition Constants**

The kinetic measurements were performed with soluble recombinant human DP IV in 0.04 M Tris/HCl buffer, pH 7.6, I = 0.125 with KCl. The K<sub>i</sub> values of inhibition of DP IV-catalyzed hydrolysis were determined using six different concentrations of Ala-Pro-pNA as substrate (10<sup>−5</sup> to 8 × 10<sup>−8</sup> M) in the absence or presence of different inhibitor concentrations around the expected K<sub>i</sub> value. The enzymatic hydrolysis of Ala-Pro-pNA was monitored at 390 nm and 30 °C on a Beckmann DU-650 UV/VIS spectrophotometer. All mixtures were started by adding the enzyme. Generally, the reaction velocities were calculated over a time interval in which less than 10% cleavage of substrate occurred. The K<sub>i</sub> values were evaluated within two independent measurements using the software Microlab origin 4.0 and SigmaPlot 5.0.

**1H NMR Spectroscopy**

NMR spectra were acquired on Bruker ARX 500, Bruker AVANCE 500, and Varian UNITY 500 instruments. Samples of 5 mg of the appropriate peptide were dissolved in 0.8 ml of H<sub>2</sub>O containing 10% D<sub>2</sub>O necessary for field frequency lock. 3-(Trimethylsilyl)-3,3,2,2-tetra-deuteropropionic acid sodium salt was used as internal standard for water samples. Water signal suppression in 1H was achieved using a pulsed 90°-delay or during relaxation delay or the 3-9-19 pulse sequence with gradients (19, 20). Spectra were acquired at 300 K. 32-1024 data points zero-filled to 64-1024 were employed to record one-dimensional spectra, whereas matrix sizes of 2<sup>1024</sup>-512 zero-filled to 2<sup>1024</sup>:1-1024 were used for two-dimensional experiments. Peaks were assigned by using H, H-COSY (21), TOCSY (22), NOESY (23), and ROESY (24). Homonuclear coupling constants were extracted from one-dimensional proton spectra using the same samples as for the two-dimensional experiments. TOCSY, NOESY, and ROESY spectra were recorded in the phase-sensitive mode (25, 26) and processed using baseline correction in both dimensions of the two-dimensional spectra. The TOCSY spectrum was carried out with the MLEV-17 sequence (27) and an 80-ms spinlock. The NOESY and ROESY experiments were carried out with the pulse sequences supplied by Bruker and Varian. The mixing times for the ROESY spectra were 50, 100, 200, 300, and 400 ms.

**Molecular Modeling**

**Structure Calculations—**The Trp<sup>2</sup>-Tat(1–9) derivative was manually built in an extended, all-trans conformation (trans-Trp<sup>2</sup>-Tat(1–9)) as well as in a conformation containing one cis peptide bond between the Trp<sup>2</sup> and the Pro<sup>3</sup> residues (cis-Trp<sup>2</sup>-Tat(1–9)). These structures were solvated by water using a precomputed water box of about 1400 solvent molecules (TIP3P water model) and were minimized to a convergence of energy gradient less than 0.001 kcal/mol × Å using the AMBER 4.1 force field (28). A distance independent dielectric constant of ε = 1 was used. For the determination of the solution conformations 18 interresidue ROEs for trans-Trp<sup>2</sup>-Tat(1–9) and 10 interresidue ROEs for cis-Trp<sup>2</sup>-Tat(1–9) obtained from the ROESY spectra were included as H-H distance restraints for molecular dynamics simulations in water. The distance restraints were applied with a force field parameter of 32 kcal/Å. The residue-based cut-off distance for nonbonded interactions was set to 10 Å. All molecular dynamics simulations were carried out using periodic boundary conditions. The starting temperature was set to 10 K, followed by slow heating to the reference temperature of 300 K. After 2 ps, the systems equilibrated at 300 K, the dynamics were performed for 500 ps, and the NTP ensemble was applied. The X-H bonds were constrained to constant values with the Shake procedure of AMBER. The time steps were 2 fs, and the nonbonded list was updated after 25 fs. The frequency of all dihedral angles particularly the dihedral angle φ of all amino acid residues was analyzed and compared with the experimental results based on the Karplus equations. Those conformations with the highest average frequency during the run time that agreed with the dihedral angles, φ, derived from the Karplus equations were used to generate solution conformations. These structures were subsequently minimized in solution. The stability of the solution conformations of trans-Trp<sup>2</sup>-Tat(1–9) and cis-Trp<sup>2</sup>-Tat(1–9) was proved by additional dynamics simulations using the same conditions described above without H-H distance restraints.

**Docking of TXA2-R(1–9) to DP IV**

A tertiary structure model of the C-terminal region, the catalytically active domain of DP IV, has been developed recently (29). Based on this model, we intended to investigate possible docking arrangements of the TXA2-R(1–9) with DP IV. A slightly modified TRIPOS (30) force field was used. The parameters epsilon of the van der Waals force field term of all carbon atoms were increased by 0.2 kcal/mol. The nonbonded cut-off was set to 16 Å. This allows the application of simulated annealing techniques without applying a huge water box surrounding the whole enzyme-ligand complex. Performing simulated annealing runs by heating the system to 700 K within 2000 fs and cooling to 100 K in 2000 fs the ligands do not move far away from the enzyme at the high temperature, but only about 10 Å on average. During the annealing phase, a multitude of stable docking conformations preferably characterized by hydrophobic interactions and strong salt bridges were obtained. Altogether, four independent simulated annealing runs with different starting arrangements, each with 50 cycles, were performed. The backbone atoms of the enzyme were kept fixed. The resulting 400 low temperature structures were saved in a data base and subsequently minimized with the standard TRIPOS force field using Gasteiger charges (31) and a distant dependent dielectric function of ε = 4r.
PBMC were prepared from heparinized blood of healthy donors as described by Reinhold et al. (32). PBMC (10^-6 cells/100 μl) were stimulated in serum-free CG medium (Vitromex) with tetanus toxoid (100 ng/ml; Calbiochem-Novabiochem, Bad Soden, Germany) in the presence of effector peptides in the concentrations indicated. After 6 days, cultures were pulsed for an additional 16 h with [3H]methionyltryptophan (0.2 μCi/ml; ICN, Eschwege, Germany). Cells were harvested onto glass fiber filters, and the incorporated radioactivity was measured by scintillation counting.

**RESULTS**

**Identification of the DP IV-inhibitory Structure in Tat(1–9)-derived Peptides**—To find out more clues about the consensus sequence of putative endogenous peptidergic DP IV inhibitors, we investigated the influence of some amino acid modifications and exchanges of the three N-terminal amino acids of the moderate DP IV inhibitor Tat(1–9) on the inhibition of DP IV. The high sensitivity and throughput of capillary electrophoresis permits the establishment of an enzymatic assay for human DP IV using IL-2(1–12), a more physiological substrate, instead of a chromogenic or fluorogenic dipeptide amide, such as Gly-Pro-pNA.

For the first screening, Tat(1–9) analogue peptides and the substrate peptide were used in equimolar concentration (400 μM). The inhibition of DP IV-catalyzed IL-2(1–12) cleavage was not significantly improved by amino acid exchanges at position 1 (methionine) or 3 (proline). Instead of enhanced DP IV inhibition, reduced DP IV inhibition was observed with peptides obtained by the installation of methionine sulfoxide or small amino acids, such as proline, glycine, or alanine, instead of methionine at the N terminus (data not shown). However, the exchange of Asp² by a series of hydrophobic as well as hydrophilic amino acids resulted in peptides with enhanced DP IV inhibitory effects (Fig. 1). In equimolar concentration to the substrate, Ala², Phe², Lys², Gly², and Ser²-Tat(1–9) inhibited DP IV-catalyzed IL-2(1–12) cleavage in the region of 70% compared with 23% for Tat(1–9). Interestingly, the Tat(1–9) peptide with tryptophan at position 2 inhibited DP IV according to a linear mixed-type mechanism, with Kᵢ = 2.12 μM (α = 16), as will be described elsewhere.² Thus, Trp²-Tat(1–9), carrying the N-terminal sequence MWP, is clearly a more potent DP IV inhibitor than Tat(1–9).

**Conformational Analysis of Trp²-Tat(1–9)**—The influence of single amino acid exchanges at position 2 of Tat(1–9) on the inhibition of DP IV-catalyzed IL-2(1–12) degradation. Original Tat sequence bears aspartic acid at position 2 and is marked with A in boldface, KIZ, benzoylcarbonyl group at the ϵ-amino group of lysine. Effector peptides and substrate were used in equimolar concentrations (400 μM). Error bars indicate S.D. of two different experiments, each carried out in triplicate.

In contrast to Tat(1–9) existing in one predominant conformation characterized by all-trans peptide bonds, the 1H NMR spectrum of Trp²-Tat(1–9) showed two major sets of signals exhibiting nearly the same population. One of these signal sets results from the cis conformer carrying a cis peptide bond between Trp² and Pro³. The presence of this cis amide bond was established based on the characteristic exchange cross peaks obtained in the two-dimensional ROESY spectrum between Val¹-C-H cis and trans, Val¹-NH cis and trans, and Asp²-NH cis and trans. Analogous to the major conformer of Tat(1–9), the second conformer of Trp²-Tat(1–9) is characterized by all-trans peptide bonds.

The theoretical conformational energy calculations were carried out using the AMBER 4.1 force field. The solution conformations of both trans-Trp²-Tat(1–9) and cis-Trp²-Tat(1–9) were determined on the basis of the NMR data. The analysis of the dynamics trajectories of the dihedral angles φ, ψ, and χ₁ gives insight into the conformational flexibility and the different structural behavior of this Tat derivative. Several solution conformations for the trans and the cis isomers that agreed with the NMR data could be determined. All vicinal coupling constants (3J₁NH-C¹H₁) and the corresponding torsion angles (φ) of this Tat derivative and the relevant interresidue ROEs suggested similar overall backbone conformations for trans-Trp²-Tat(1–9) and cis-Trp²-Tat(1–9).

The exchange of Asp² with the hydrophobic tryptophan does not cause a significant rearrangement of the backbone structure of Trp²-Tat(1–9) compared with Tat(1–9) (Fig. 2). The superpositions of the determined solution conformations of both trans and cis isomers of Trp²-Tat(1–9) show a relatively rigid structure along the residues Pro³ and Pro⁶. There are remarkable similarities between the solution conformations of

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² S. Lorey, A. Stöckel-Maschek, J. Faust, W. Brandt, B. Stiebitz, M. Gorrell, T. Kähne, S. Wrenger, D. Reinhold, S. Ansorge, and K. Neubert, manuscript in preparation.
both isomers (all-trans-Trp²-Tat(1–9) and cis-Trp²-Tat(1–9)) and Tat(1–9). In conclusion, the highly improved effect of Trp²-Tat(1–9) on DP IV inhibition cannot be explained by a significantly altered backbone structure of this analogue compared with that of Tat(1–9).

Identification of the N Terminus of the Thromboxane A2 Receptor as a Putative DP IV Inhibitor—The search for proteins extracellularly exposing the N-terminal XWP sequence using the Swiss-Prot protein database revealed a single hit, the thromboxane A2 receptor (TXA2-R) sequence. TXA2-R belongs to the G-protein-coupled receptor family. Because the N terminus is localized extracellularly, it is conceivable that TXA2-R is an endogenous DP IV inhibitor. To prove whether this holds true, we studied the influence of the N-terminal nonapeptide of TXA2-R, TXA2-R(1–9), on the enzymatic activity of DP IV and on the proliferation of stimulated PBMC. Indeed, TXA2-R(1–9) (MWPNGSSLG) inhibited DP IV-catalyzed IL-2(1–12) cleavage as potent as Trp²-Tat(1–9) (Fig. 3A). Compared with both Tat(1–9) and Trp²-Tat(1–9), TXA2-R(1–9) inhibits DP IV competitively. The $K_i$ value of 5.06 μM for TXA2-R(1–9) determined using Als-Pro-pNA and soluble human DP IV, is in the same range as the $K_i$ value of Trp²-Tat(1–9). Moreover, the reduction of DNA synthesis in tetanus toxoid-stimulated PBMC by TXA2-R(1–9) is as strong as by Trp²-Tat(1–9), which is in striking correlation with the effects of both peptides on DP IV activity (Fig. 3B). This indicates a DP IV-mediated suppressive effect on DNA synthesis of stimulated immune cells by the N-terminal nonapeptide of TXA2-R.

To address the question of whether the suppressive effect of DP IV-inhibitory peptides on DNA synthesis of tetanus toxoid-stimulated PBMC correlates with a decrease in production and secretion of cytokines, we measured the concentrations of IL-2 in supernatants of tetanus toxoid-stimulated PBMC in the presence of Tat(1–9), Trp²-Tat(1–9), and TXA2-R(1–9). Interestingly, as shown in Fig. 4, the IL-2 production was significantly reduced by all three DP IV inhibitors at concentrations of 20 μM after 24 h.

To examine whether the immunoinhibitory cytokine TGF-β1 is involved in the DP IV inhibitor-induced suppression of DNA synthesis and IL-2 production, the concentrations of this cytokine in supernatants of tetanus toxoid-stimulated PBMC were assayed in the presence or absence of DP IV inhibitors. Interestingly, Tat(1–9), Trp²-Tat(1–9), and TXA2-R(1–9) were capable of inducing a 2-fold increase in secretion of latent TGF-β1 on tetanus toxoid-stimulated PBMC after a period of 24 h (Fig. 4).

Moreover, to explain the enhanced inhibitory potential of nonapeptides bearing tryptophan at position 2, we investigated the docking behavior of TXA2-R(1–9) to the active site of DP IV. For this investigation, knowledge of the tertiary structure of DP IV is necessary. Because the x-ray structure of DP IV has not been solved as yet, we used a model of the active site-containing C-terminal domain (residues 502–766) of human DP IV, developed based on the x-ray structure of the DP IV-related enzyme prollyl oligopeptidase (29, 37) (Fig. 5A). In homology to prollyl oligopeptidase, the S1 binding pocket for proline residues of DP IV substrates is formed by Tyr²⁶₆, Tyr³³¹, and Val⁶⁶⁵. Applying a simulated annealing procedure with modified force field parameters, several low energy docking arrangements could be detected, the most stable of which is represented in Fig. 5B. The salt bridge between the protonated N terminus of TXA2-R(1–9) and the side chain of Glu⁶⁶⁸ of DP IV is formed in accordance with DP IV substrates (29). Two alternative attractive interactions were observed for Trp² of TXA2-R(1–9): the formation of a sandwich-like interaction with the aromatic ring of Tyr²⁶₆ of DP IV shown in Fig. 5B, and the interaction with the side chain of Val⁶⁶⁵, which is also close to Trp². In each case, the strong hydrophobic interactions between these residues may explain the importance of Trp² of peptides being DP IV inhibitors.

**DISCUSSION**

Evidence exists that the mechanisms of DP IV action in the immune system are rather complex. DP IV processes a number of chemokines including RANTES (regulated on activation normal T cell expressed and secreted) and SDF-1 (stromal cell-derived factor-1) generating naturally occurring truncated peptides with a significantly altered receptor specificity and thus biological activity (38). Recently, the N-terminal truncation of another oligopeptide, procalcitonin, an established clinical marker for bacterial sepsis, by DP IV has been demonstrated (39). However, the cleavage of immunologically relevant substrates is probably not the only feature of DP IV/CD26 important for its function in the immune system. Experiments with synthetic DP IV inhibitors clearly pointed out that enzymatically active DP IV contributes as an accessory protein to the signaling of the T cell receptor-CD3 complex and induces T cell proliferation (4). Thus, DP IV has been discussed as a candidate for generating a costimulatory signal for T cell activation. Moreover, DP IV is a binding partner for different proteins, e.g. adenosine deaminase, the phosphatase CD45, and the HIV-1 transactivator Tat, which are not substrates of DP IV (12, 40, 41).

HIV-1 Tat is described as the first known natural inhibitor of DP IV (12, 13) and it suppresses antigen-, anti-CD3-, and mitogen-induced activation of human T cells (7, 11). Interestingly, Tat contains N-terminal XXP and the short N-terminal nonapeptide of Tat (Tat(1–9)), also inhibits DP IV, and interferes with the proliferation of tetanus toxoid-stimulated PBMC (8).

Here, to gain further information about the structure in Tat(1–9), which mediates both the inhibition of DP IV and the suppression of DNA synthesis, we examined the influence of modifications and of single amino acid exchanges at the first three positions of the Tat(1–9) amino acid sequence. The cen-
Interaction of the N-terminal Part of TXA2-R with DP IV/CD26

Fig. 3. Dose-dependent inhibition of DP IV-catalyzed IL-2(1–12) cleavage (A) and of DNA synthesis of tetanus toxoid-stimulated PBMC (B) by Tat(1–9), Trp2-Tat(1–9), and TXA2-R(1–9). A, error bars indicate S.D. of two different experiments, each carried out in triplicate. B, PBMC (10^6 cells/ml) were incubated with tetanus toxoid (100 ng/ml) in the presence of effector peptides at the concentrations indicated. After 6 days, cultures were pulsed for additional 16 h with [3H]methylthymidine incorporation in control cultures, 8400 ± 1300 cpm. The one-letter amino acid code is used.

FIG.4. Influence of Tat(1–9), Trp2-Tat(1–9), and TXA2-R(1–9) on production of IL-2 and latent TGF-β1 in tetanus toxoid-stimulated PBMC. PBMC (10^6 cells/ml) were incubated with tetanus toxoid (100 ng/ml) and the DP IV effectors (20 μM). After 24 h, supernatants were harvested and stored at −70 °C. The IL-2 and TGF-β1 concentrations were measured with enzyme immunoassays. Results are shown as mean ± SD from three independent experiments. The values are expressed as percentage of cytokine production in relation to control cultures without DP IV inhibitor (cytokine production in control cultures, 136 ± 45 pg/ml for IL-2 and 1.4 ± 0.7 ng/ml for TGF-β1). The one-letter amino acid code is used.

The ability of synthetic highly specific DP IV inhibitors in vitro to modulate immune cell activation suggests the existence of endogenous peptides using this method of immunoregulation. Using database searches for N-terminal XWP, we found the TXA2-R sequence. TXA2-R is a G protein-coupled receptor with seven putative transmembrane helices locating a relatively long MWP-bearing N terminus (29 amino acids) at the outer site of the plasma membrane (48). Interestingly, N-terminal epitope tagging of TXA2-R did not alter ligand affinities, nor did it influence inositol phosphate generation in response to a TXA2-R agonist (49). This suggests that the N-terminal region is not involved in ligand binding and signaling and does not undergo rigid interactions with the extracellular loops but enters a relatively flexible structure. Thus, the extracellular N-terminal region of TXA2-R should be accessible to interactions with the catalytic domain of DP IV. Indeed, the N-terminal nonapeptide of TXA2-R, TXA2-R(1–9), inhibits DP IV. The inhibition is nearly as strong as that by Tat(1–9), stressing the importance of the MWP motif for DP IV inhibition, because the following six amino acids of the TXA2-R sequence are completely different from the Trp 2-Tat(1–9) analogue can only be due to the presence of tryptophan in the second position, suggesting that the side chain of tryptophan is more favored to exhibit attractive interactions with DP IV compared with aspartic acid.

Using synthetic inhibitors, the viral polypeptide Tat, or other XXP-peptides, it has been demonstrated that DP IV plays an important role in the activation, including cytokine production and proliferation of lymphocytes in vitro (6, 7, 32, 43). A series of in vivo studies with different specific DP IV inhibitors supports the physiological relevance of these processes. Subcutaneously administered Pro-boroPro suppresses antibody production in mice immunized with bovine serum albumin (44). Alkylpindamine-induced arthritis in rats, a model for rheumatoid arthritis, is suppressed by several DP IV inhibitors, i.e. AlaboroPro, Lys[Z(NO2)]-thiazolidide, and Ala-Pro-nitrobenzoyl-hydroxylamine (45). The inhibition of DP IV with prodipine abrogates acute rejection of cardiac allografts in rats and prolongs allograft survival from 7 to 14 days, demonstrating the role of CD26/DP IV in alloantigen-mediated immune regulation in vivo (46). Recently, a protective and therapeutic effect of the DP IV inhibitor Lys[Z(NO2)]-pyrrolidide in experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis, has been reported; this finding identifies DP IV/CD26 as a possible drug target in inflammatory autoimmune diseases (47).

The central result is that aspartic acid at position 2 is rather unfavorable, and its exchange with tryptophan results in a peptide with strongly enhanced effects on DP IV inhibition and on DNA synthesis suppression. The inhibitory effect of Trp2-Tat(1–9) is in the range of those of the inhibitors TMC-2A and TSL-225, (Ki values of 5.06 and 3.6 μM, respectively, compared with 2.12 μM for Trp2-Tat(1–9)), which exert anti-inflammatory effects on experimental arthritis in rat (42). In conclusion, the N-terminal motif XWP turned out to be important for DP IV inhibition.

Conformational analysis by NMR spectroscopy in conjunction with restrained molecular dynamics simulations indicates that the exchange of Asp^2 with the hydrophobic tryptophan residue does not cause a significant rearrangement of the backbone structure of Trp^2-Tat(1–9) in comparison to that of Tat(1–9), which was solved already (8, 35, 36). Thus, the considerable enhancement of inhibition capacity of the Trp^2-Tat(1–9) analogue can only be due to the presence of tryptophan in the second position, suggesting that the side chain of tryptophan is more favored to exhibit attractive interactions with DP IV compared with aspartic acid.

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with its specific ligands on antigen-presenting cells (e.g., CD2-CD58, CD28-CD80, and CD11a/CD18-CD54), the contact between both cells becomes very intensive. On the basis of this knowledge and the topology of TXA2-R, it is highly probable that during antigen presentation the N-terminal part of TXA2-R is able to interact with DP IV/CD26 highly expressed on T cells. Inhibition of DP IV activity by synthetic inhibitors or XXP peptides leads to suppression of DNA synthesis and of production of immunostimulatory cytokines, such as IL-2 and interferon-γ (4). Here, we demonstrate that TXA2-R(1–9) also suppresses DNA synthesis, as well as IL-2 production of tetanus toxoid-stimulated PBMC. This indicates that TXA2-R could be an endogenous ligand of DP IV modulating T cell activation via DP IV inhibition, as was previously observed for synthetic DP IV inhibitors (4).

The molecular mechanisms contributing to the immunosuppressive effects mediated by different DP IV inhibitors have not been elucidated in detail yet. However, it is well established that inhibition of DP IV finally induces production and secretion of the immunosuppressive cytokine TGF-β1 (4, 5, 43). The released TGF-β1 itself is known to induce a cell cycle arrest (52) associated with the suppression of proliferation and of production of different cytokines. The finding that TXA2-R(1–9) enhances the TGF-β1 production in tetanus toxoid-stimulated PBMC demonstrated that it also acts via the above described common pathway marked by DP IV inhibition and following TGF-β1 production. This confirms the importance of the N-terminal MWP motif for CD26-mediated suppression of immune cell activation.

In conclusion, by amino acid exchanges based on the sequence of the moderate DP IV inhibitor Tat(1–9), we identified Trp2-Tat(1–9) inhibiting DP IV clearly more efficiently. Subsequently, we found the G-protein-coupled receptor TXA2-R bearing the same sequence, MWP, at the extracellularly localized N terminus. The topology of the N-terminal MWP sequence and its localization on antigen-presenting cells, namely monocytes, raise the possibility that the N-terminal part of TXA2-R might be an endogenous DP IV inhibitor and contribute to the limitation of the immune response. During antigen presentation the TXA2-R/DP IV interaction could result in TGF-β1-mediated down-regulation of the T cell activation as a negative feedback mechanism. The experimental data presented here provide the first indication of such a function of the N-terminal part of TXA2-R. Additional experiments will be needed to prove this interesting hypothesis.

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