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Inhibition of dipeptidyl peptidase IV (DP IV) by anti-DP IV antibodies and non-substrate X-X-Pro- oligopeptides ascertained by capillary electrophoresis

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Abstract

Dipeptidyl peptidase IV (DP IV)-catalyzed hydrolysis of the NH_2-X-Pro-containing N-terminal dodecapeptide of IL-2 was studied using free zone capillary electrophoresis as an alternative peptidase assay. In contrast to the conventional DP IV substrate glycyl-prolyl-*p*-nitroanilide (Gly-Pro-pNA), the hydrolysis of this peptide by DP IV was found to be significantly inhibited by anti-DP IV antibodies. Inhibition of DP IV was also observed with a number of non-substrate oligopeptides containing an N-terminal X-X-Pro- structure, including the HIV Tat protein. For Met-IL-2(1–6), we determined a competitive inhibition with an inhibition constant of ca. 100 μM.

1. Introduction

Dipeptidyl peptidase IV (DP IV, CD26, E.C. 3.4.14.5) is an exopeptidase localized on the surface of leukocytes. DP IV cleaves dipeptides with an X-Pro or X-Ala dipeptide sequence from the N-terminal part of peptides. Peptides containing proline or hydroxyproline in P1′-position will not be hydrolyzed by this enzyme [1].

Our data and that of other groups indicate that this membrane-bound exopeptidase is involved in the regulation of lymphocyte activation and immune response [2–15]. Most of these results were obtained from investigations with synthetic inhibitors, the microbial diprotins or anti-DP IV antibodies. A number of peptides originating from the neuroendocrine system (e.g. substance P, β-casomorphin, neuropeptide Y, peptide YY, growth hormone releasing factor) were shown to be substrates of DP IV [16–21]. Callebaut et al. [22] reported that DP IV is involved in the HIV infection of T cells by functioning as a coreceptor molecule for the virus. It is postulated that HIV binds via the V3-loop of the gp120 molecule to DP IV. Furthermore, the HIV Tat protein is known to be an inhibitor of DP IV [23]. However, in the immune system, the physiological substrates or effectors of this enzyme are unknown, so far.

Recently, using capillary free zone electrophoresis, we showed that DP IV, alone or in combination with the aminopeptidase N, is ca-

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pable of hydrolyzing oligopeptides analogous to the N-terminal structure of different cytokines (IL-1β, IL-2, IL-6, TNF-β). The hydrolysis rates of these oligopeptides were affected by their chain length and glycosylation [24,25]. However, intact cytokines were apparently no substrates of these exopeptidases.

Applying capillary electrophoresis, we searched for peptides and antibodies capable of influencing the DP IV-catalyzed oligopeptide hydrolysis. We studied the effects of one polyclonal and different monoclonal anti-DP IV antibodies on DP IV-catalyzed hydrolysis of IL-2(1–12), in comparison with the cleavage of the low-molecular-mass DP IV substrate Gly-Pro-pNA. Whereas Gly-Pro-pNA hydrolysis was not influenced by any of these antibodies, the hydrolysis of the oligopeptide was inhibited by two of the six investigated antibodies in a dose-dependent manner.

Moreover, we show that HIV gp120 as well as different synthetic peptides analogous to the V3-loop of gp120 have no inhibitory effects on DP IV-catalyzed hydrolysis of oligopeptides, whereas the HIV Tat protein and a number of non-substrate peptides with similar N-terminal X-X-Pro- sequences significantly inhibit the cleavage of oligopeptides as well as the degradation of Gly-Pro-pNA by DP IV in a competitive manner.

2. Experimental

2.1. Chemicals

Dipeptidyl peptidase IV (porcine kidney) was kindly provided by Dr. U. Demuth (Department of Biochemistry/Biotechnology, University Halle-Wittenberg).

The monoclonal anti-DP IV antibodies EF5/A3, PEG2/C3, EF6/B10, PEG2/G11, and EF6/F11 were produced in our laboratory using $CD26^+$ U937 cells for immunization. For production of the polyclonal goat-anti-DP IV antibody purified DP IV from pig kidney was used for immunization.

IL-2(1–12), Met-IL-2(1–6), and Met-IL-2(1–12) were synthesized by solid-phase peptide synthesis with Fmoc technique using the peptide synthesizer 431A (Applied Biosystems). The HIV Tat protein was synthesized on polyoxyethylene–polystyrene graft resin in a continuous-flow instrument constructed and operated as described by Frank and Gausepohl [26]. Peptide chain assembly was performed using Fmoc chemistry [27] and in situ activation of amino acid building blocks by PyBOP [28]. The synthesized peptides were purified by reversed-phase HPLC and characterized by mass spectrometry.

Gp120 and its subpeptides HBX-2, NY/5, and V3-loop(313–320) were purchased from ABT (London, UK). Peptide YY(3–36), gastrin releasing factor(17–24), tuftsin, substance P(2–11), Met-Lys-Bradykinin and the tetrapeptides GGPA and AAPA as well as the Gly-Pro-pNA were obtained from Bachem (Heidelberg, Germany).

For enzymatic assays all compounds were diluted in a 0.01 *M* sodium phosphate buffer pH 7.4.

2.2. DP IV-catalyzed hydrolysis of oligopeptides

Influences of peptides or antibodies on DP IV-catalyzed oligopeptide hydrolysis were tested using IL-2(1–12) as substrate. A 2-μl aliquot of peptide solution or buffer, respectively, was added to 0.5 μl of DP IV (550 pkat/ml). The enzymatic reaction was started by addition of 2 μl of a 1 m*M* substrate stock solution. Final concentration of substrate was 400 μM. Samples were incubated for 30 min at 37°C. Thereafter the reaction was stopped by addition of 2 μl of 0.03 *M* phosphoric acid containing 500 μM histidine as an internal standard for capillary electrophoresis.

2.3. Capillary electrophoresis

Degradation of the IL-2(1–12) was measured by capillary free zone electrophoresis using the Biofocus 3000 system of Bio-Rad. Separations were performed under following conditions: capillary, 24 cm total length, 20 cm effective

length, 25 μm I.D., 125 μm O.D., coated (Bio-Rad, Munich, Germany); injection, pressure 1.03 MPa s; run, 14 kV constant voltage, positive to negative, 0.1 *M* sodium phosphate buffer pH 2.5 with linear polymer (Bio-Rad, Munich, Germany), current in the range of 20 μA; detection, UV 200 nm; capillary temperature, 15°C; carousel temperature, 10°C.

2.4. Hydrolysis of Gly-Pro-pNA

DP IV-catalyzed hydrolysis of Gly-Pro-pNA was measured photometrically using the Cary 1 spectrophotometer (Varian, Darmstadt, Germany). DP IV-activity was determined by measurement of the rate of *p*-nitroaniline generation monitored at 392 nm.

2.5. Determination of inhibition type and inhibitory constant

For the heptapeptide Met-IL-2(1–6), inhibition type and inhibitory constant (K_i) were determined using Gly-Pro-pNA as substrate. We measured the rate of Gly-Pro-pNA cleavage at four substrate concentrations in the range of 20 to 200 μ*M* using inhibitor concentrations in the range of 5 to 400 μ*M*. Inhibition type and K_i were calculated with the help of a Dixon plot.

3. Results

DP IV-catalyzed peptide hydrolysis can be measured by the classical method using chromogenic substrates (e.g. Gly-Pro-pNA) or by studying the degradation of oligopeptides containing an N-terminal X-Pro- sequence. In comparison to the spectrophotometrical measurement of DP IV activity, capillary electrophoresis provides the opportunity of investigating potential physiological peptide substrates of different origin. In Fig. 1 the electrophoretical detection of IL-2(1–12) degradation by DP IV is shown (A and B). Effects of a non-inhibitory (C) and an inhibitory peptide (D) on this hydrolysis are also demonstrated.

3.1. Inhibition of DP IV-catalyzed hydrolysis of oligopeptides by antibodies

The influence of the monoclonal anti-DP IV antibodies EF5/A3, PEG2/C3, EF6/B10, PEG2/G11, and EF6/F11 and a polyclonal goat-anti-DP IV antibody was studied by means of the DP IV-catalyzed hydrolysis of oligopeptides as well as the hydrolysis of the Gly-Pro-pNA. We found that hydrolysis of oligopeptides is inhibited by the polyclonal antibody and by the monoclonal PEG2/G11 in a dose-dependent manner (Fig. 2). In contrast to this, all investigated antibodies were not capable of inhibiting the hydrolysis of the low-molecular-mass substrate Gly-Pro-pNA. The observed increase of the Gly-Pro-pNA hydrolysis rate by antibodies seems to be nonspecific because addition of irrelevant mouse immunoglobulin or serum bovine albumin had similar effects (Fig. 2).

3.2. Inhibition of DP IV activity by non-substrate peptides

Starting from the hypothesis of Callebaut et al. [22] that DP IV is a coreceptor of the HIV-virus, we looked for inhibitory effects of the gp120 and some V3-loop peptides on the DP IV-catalyzed oligopeptide hydrolysis. Under the conditions used, we could not find any significant effects of one of the tested gp120 peptides on this enzymatic activity (Table 1, Fig. 3). On the other hand, the DP IV-catalyzed oligopeptide hydrolysis was inhibited by the HIV Tat protein. This is in accordance with the results of Gutheil et al. [23] who found an inhibition of Ala-Pro-pNA hydrolysis by the Tat protein. Based on this result, we studied the effects of other peptides which have similar N-terminal structures, i.e. proline in the third position (Table 1). Tetrapeptides with the structures GGPA, AAPA, and TKPR were found to have no inhibitory effects. In contrast longer peptides were shown to exhibit significant inhibition (Table 1, Fig. 3). The highest inhibition was measured with the Tat protein. Hydrolysis of the inhibitory peptides themselves could be excluded by capillary electrophoresis. All peptides with an X-X-Pro se-

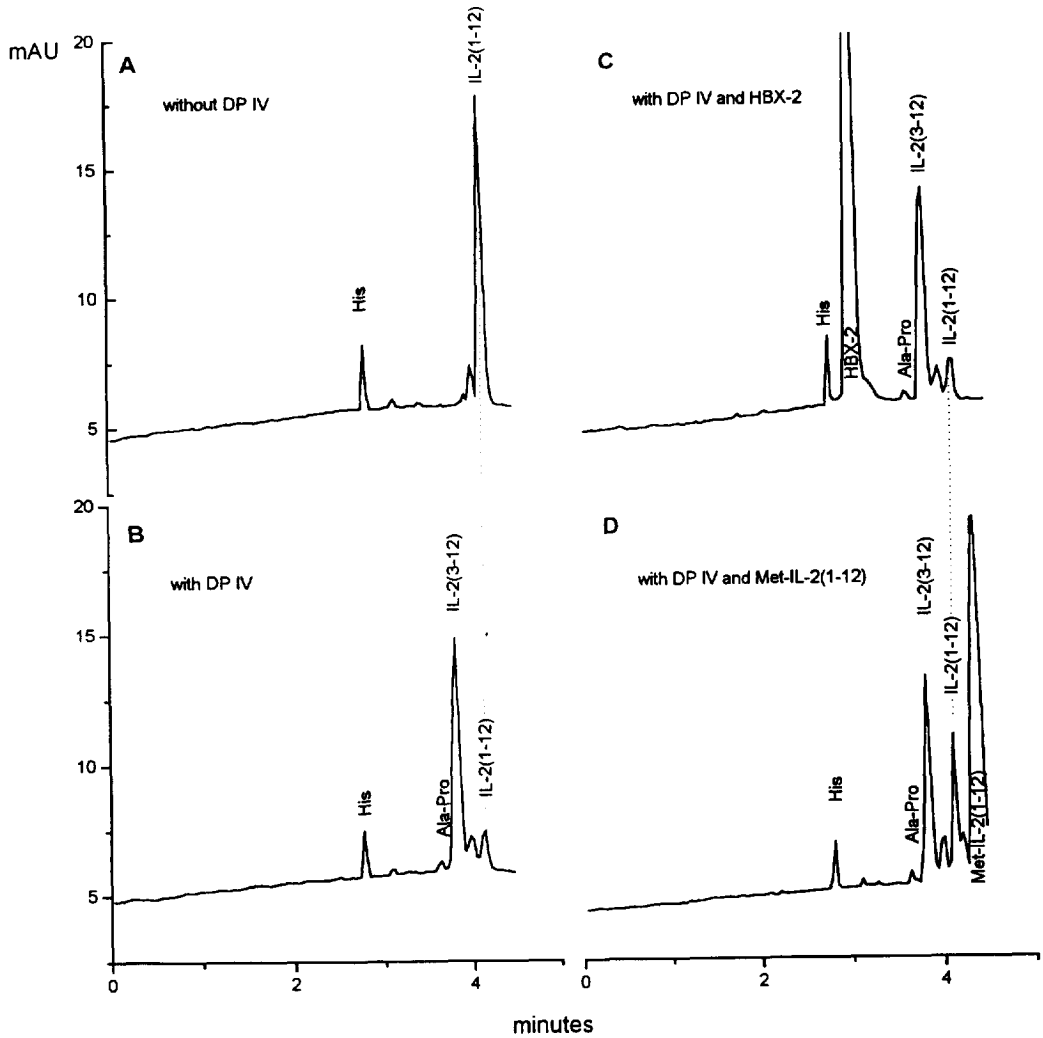

Fig. 1. Detection of DP IV-catalyzed hydrolysis of IL-2(1–12) by capillary electrophoresis. Electropherograms show IL-2(1–12) and its cleavage products after 30 min of incubation at 37°C without DP IV (A), with DP IV alone (B), with DP IV and the non-inhibitory peptide HBX-2 (C), and with DP IV and the inhibitory peptide Met-IL-2(1–12) (D). Concentrations of the substrate and the additional peptides were 400 μM and of DP IV 60 pkat/ml. Histidine was used as an internal standard.

quence were resistant against treatment by DP IV for several hours. Thus, the inhibitory effect of the X-X-Pro- peptides cannot be explained by competitive cleavage of these peptides.

3.3. Determination of inhibitor type and inhibition constant

The kinetic characterization of the inhibition of DP IV by Met-IL-2(1–6) was studied in more detail using Gly-Pro-pNA as substrate. The point of intersection in the second quadrant of the Dixon plot indicates that DP IV is inhibited competitively by this X-X-Pro- peptide. The x-value of the intersection represents the negative inhibition constant, which is ca. 100 μM.

4. Discussion

DP IV is an exopeptidase localized on the surface of lymphocytes. This molecule plays an

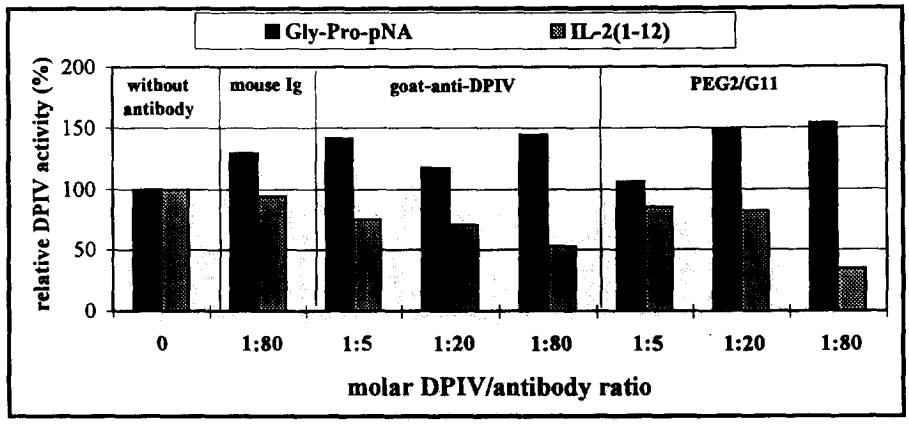

Fig. 2. Comparison between the effects of antibodies (goat-anti-DP IV, PEG2/G11) on DP IV-catalyzed hydrolysis of Gly-Pro-pNA and IL-2(1–12). Investigations were done with substrate concentrations of 400 μM. In both enzyme assays the same DP IV/antibody ratios were used. The DP IV concentrations for oligopeptide hydrolysis were 0.44 μg/ml and for Gly-Pro-pNA cleavage 0.013 μg/ml ($n = 3$)

important role in the regulation of lymphocyte activation and immune response. Investigations with synthetic inhibitors showed that the enzymatic activity of DP IV is involved in these processes [2,4,7,9,29]. A number of antibodies (e.g. Ta1, 1F7) with different, in part contrary, effects on the activation of lymphocytes were designed [5,7,30–36]. None of the antibodies studied so far was found to have inhibitory effects on the enzymatic activity of DP IV. Using capillary electrophoresis to measure DP IV-catalyzed oligopeptide hydrolysis, we found that two of the six antibodies investigated in this study are capable of inhibiting DP IV activity. The differences in the effects of antibodies on DP IV-catalyzed hydrolysis of oligopeptides could be explained by different epitope specificity. Interestingly, hydrolysis of the low-molecular-mass

Table 1
Sequence and length of oligopeptides investigated for their inhibitory effects to DP IV-catalyzed oligopeptide hydrolysis

	Peptide	N-Terminal structure	Number of amino acids	Inhibition of DP IV activity[a]
HIV gp120 peptides	V3-loop(313–320)	RIQRGPR	7	–
	NY/5	CNTKKGIAIGPG...	20	–
	HBX2	CNTRKRIRIQRGPG...	22	–
	gp120	TEKLWVTVYYGV...	477	–
X-X-Pro-peptides	GGPA	GGPA	4	–
	AAPA	AAPA	4	–
	Tuftsin	TKPR	4	–
	Met-IL-2(1–6)	MAPTSSS	7	++
	Substance P(2–11)	PKPQQFFGLM	10	+
	Met-IL-2(1–12)	MAPTSSSTK...	13	++
	GRF(17–24)	MYPRGNHW...	14	++
	Peptide YY(3–36)	IKPEAPGEDA...	34	++
	Tat protein	MDPVDPNIEP...	86	+++
	Met-Lys-Bradykinin	MKRPPGFSPFR	11	–

[a] – = no inhibition; + = weak inhibition; ++ = medium inhibition; +++ = strong inhibition.

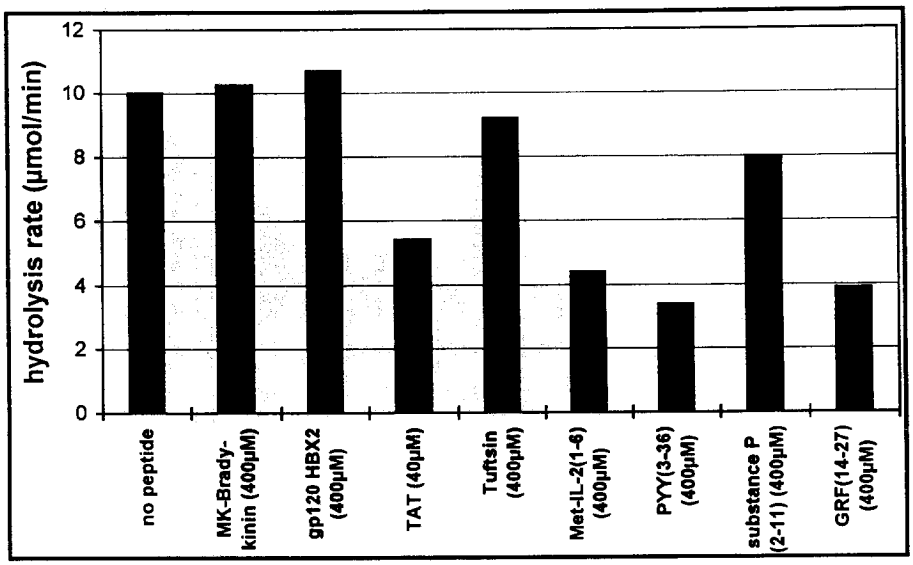

Fig. 3. Effects of different non-substrate peptides on DP IV-catalyzed hydrolysis of IL-2(1–12). DP IV-catalyzed hydrolysis of IL-2(1–12) was determined as described in the Experimental section. Met-Lys-Bradykinin was used as an negative control for peptide effects. All peptides with exception of the Tat protein were used in a concentration of 400 μM. The concentration of the Tat protein was 40 μM ($n = 3$)

Gly-Pro-pNA was not affected by one of these antibodies. This suggest that the inhibitory antibodies, goat-anti-DP IV and PEG2/G11, bind near the active site of the DP IV, resulting in a sterical hindrance of the binding of longer peptides. An other explanation for the reduced accessibility of the active center could be a conformational change of the DP IV molecule as a consequence of antibody binding. These results clearly demonstrate that the use of small DP IV substrates such as Gly-Pro-pNA is of limited value, and alternative approaches using oligopeptides as substrates and suitable detection techniques, e.g. capillary electrophoresis, are necessary for the study of the physiological role of DP IV and other peptidases.

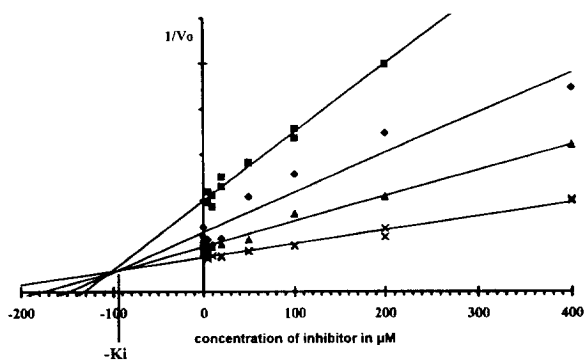

Fig. 4. Determination of inhibition type and inhibition constant of Met-IL-2(1–6) by a Dixon plot. Substrate concentrations (Gly-Pro-pNA) of 20, 50, 100, and 200 μM were used. The DP IV activities were determined at eight concentrations of the inhibitor Met-IL-2(1–6) (up to 500 μM) at each substrate concentration. Measurements were done with a DP IV concentration of 13 ng/ml.

Adenosine deaminase [37], HIV gp120 [22], and HIV Tat protein [23] are known as natural effectors of DP IV. Using the method described here, we could show that gp120 as well as some V3-loop peptides do not effect the DP IV activity in vitro. However, the Tat protein was found to show a strong inhibition of the DP IV-catalyzed hydrolysis of oligopeptides. Moreover a number of non-substrate peptides characterized by an N-terminal X-X-Pro- sequence, were also found to be capable of inhibiting DP IV activity. From the competitive type of inhibition, we can conclude that these peptides may represent a new type of DP IV inhibitor which is capable of binding to the active site of this enzyme but which cannot be cleaved. The extend of inhibition of these peptides obviously depends on their

length. Smaller tetrapeptides don not show inhibitory effects, whereas the Tat protein, consisting of 86 amino acids, was the most potent inhibitor among the peptides tested. The effect of Tat is comparable with the inhibition of DP IV by diprotin A, a tripeptide with proline in the second position [38,39]. The DP IV inhibitors used so far are X-Pro dipeptide analogs where proline is replaced by the amino boronic acid of proline [29], by thiazolidide, or by pyrrolidide [9,40]. These are more potent inhibitors than the X-X-Pro- peptides investigated in this study. However, the X-X-Pro- peptides evoke our interest because a number of natural peptides contain this N-terminal structure. It is conceivable that the DP IV activity is regulated by such peptides in vivo. Also the in vivo effects of the HIV Tat protein could be based on a deregulation of DP IV-mediated growth processes. Other interesting candidates are neuropeptide Y and the peptide YY, because they are substrates of DP IV and cleavage products characterized by the N-terminal X-X-Pro- sequence might suppress the enzymatic activity of this enzyme.

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Abbreviations

DP IV	Dipeptidylpeptidase IV
Gly-Pro-pNA	Glycyl-prolyl-*p*-nitroanilide
HBX-2	gp120 V3-loop peptide
HIV	Human immunodeficiency virus
IL	Interleukin
IL-2(1–12)	Synthetic N-terminal dodecapeptide of IL-2
IL-2(1–6)	Synthetic N-terminal hexapeptide of IL-2
K_i	Inhibition constant
NY/5	gp120 V3-loop peptide
PyBOP	Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium-hexafluorophosphate
TNF-β	Tumor necrosis factor β
U937	Human histiocyte cell line

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