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Dipeptidyl peptidase IV (CD 26) and aminopeptidase N (CD 13) catalyzed hydrolysis of cytokines and peptides with N-terminal cytokine sequences

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A number of natural cytokines are characterized as having dipeptidyl peptidase (DP) IV susceptible N-terminal peptide sequences. Here we demonstrate that oligopeptides with sequences analogous to the N-terminal part of human IL-1β, IL-2, TNF-β and murine IL-6 were hydrolyzed by purified DP IV and aminopeptidase N (AP-N). The rate of DP IV-catalyzed hydrolysis of these peptides was negatively correlated with their chain length. In contrast to these results, no degradation was found under our conditions for the intact recombinant cytokines, IL-1α, IL-1β, IL-2, G-CSF and for natural IL-2, independent of whether DP IV and AP-N were used separately or in combination.

Dipeptidyl peptidase IV; Aminopeptidase N; Cytokine; Cytokine partial sequence

1. INTRODUCTION

Recent data in the literature have provided evidence that proteolytic enzymes are involved in the regulation of release [1–4], modification [5–8], and inactivation [9] of cytokines.

Special interest has been focussed on peptidases which are localized on the plasma membrane of immune cells, e.g. dipeptidyl peptidase IV (DP IV, CD26, EC 3.4.14.5) and aminopeptidase N (AP-N, CD13, EC 3.4.11.2), because these ectopeptidases are candidates for direct interaction with cytokines and their receptors. DP IV is an exopeptidase releasing dipeptides with a X-Pro(Ala) sequence from the N-terminal part [10]. AP-N is able to split off amino acids from the N-terminus, with the exception of proline in the penultimate position [11]. Data from our and other laboratories have shown that these membrane-bound exopeptidases are involved in the regulation of lymphocyte activation and immune response [12–20]. However, their physiological substrates in the immune system are as yet unknown.

Interestingly, many of natural cytokines, e.g. IL-1β, IL-2, IL-3, IL-6, TNF-β, GM-CSF and G-CSF, have N-terminal sequences with proline in the second position. In the case of other cytokines, e.g. IL-1α, interferons and recombinant cytokines, proline is localized in the third or later position of the N-terminus. In these cases, a joint action of both AP-N and DP IV could theoretically be possible for the removal of the X-Pro-sequence from the N-terminus or modification of the N-terminal part of these cytokines, respectively.

In this context it is of interest that the biological activity of IL-1α, IL-1β and IL-2 is changed strongly by short deletions or mutations at their N-terminal part [21–23]. For IL-1β it was shown that deletion of the four N-terminal amino acids leads to conformational changes and a lost of receptor binding capability as well as biological activity [24,25].

In the current work we have investigated the capability of DP IV and/or AP-N to modify the N-terminal part of some cytokines with susceptible sequences. We report here data showing that a number of synthetic oligopeptides with sequences analogous to the N-terminal part of natural and recombinant cytokines are degraded by DP IV or by a combined action of AP-N and DP IV. We also show, however, that the intact cytokines, rIL-1α, rIL-1β, rIL-2, nIL-2 and G-CSF, are resistant to the action of these peptidases under in vitro conditions.

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Abbreviations: AP-N, aminopeptidase N; DP IV, dipeptidyl peptidase IV; Fmoc, fluorenylmethoxycarbonyl; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; g, glycosylated; mIL, murine interleukin; MNC, mononuclear cells; nIL, natural interleukin; POE, polyoxyethylene; rIL, recombinant interleukin; TGF-β, transforming growth factor; TNF, tumour necrosis factor.

2. MATERIALS AND METHODS

2.1. *Enzymes and substrates*

DP IV (porcine kidney) was kindly provided by Dr. U. Demuth (Department of Biochemistry and Biotechnology, University of Halle-Wittenberg). AP-N was purified from human kidney according to the method described for DP IV by Rahfeld et al. [26] with an additional affinity purification step using rabbit anti-AP-N antibodies coupled to cyanogen bromide activated Sepharose 4B.

Human IL-1β(1–6), IL-2(1–6), IL-2(1–12), IL-2(1–24), Met-IL-2(1–6), Met-IL-2(1–12), lymphotoxin(1–5), murine IL-6(1–12) and murine IL-6(1–12)-polyoxyethylene (POE) were synthesized by solid-phase peptide synthesis by the Fmoc technique using peptide synthesizer 431A (Applied Biosystems). An additional human IL-2(1–24) was provided by Dr. Conradt (Gesellschaft für Biotechnologische Forschung, Braunschweig). O-Glycosylated IL-2 tetra-, hexa-, octa- and decapeptides (2-acetamido-2-desoxy-D-galactoseamine at Thr^3) were obtained from Prof. H. Paulsen (Department of Organic Chemistry, University of Hamburg).

Recombinant IL-1β was a product of Pharma Biotechnologie Hannover. Recombinant IL-1α and recombinant IL-2 were produced by LaRoche, Basel. Natural IL-2 was kindly provided by Dr. J. Knüver-Hopf (DRK-Blutspendedienst Niedersachsen, Springe). Recombinant G-CSF was a kind gift of Prof. H. Welte (Medical School, Hannover).

2.2. *Hydrolysis of peptides by DP IV and/or AP-N*

Peptides (50 μM) were incubated with DP IV (2.6 mU/ml) in 0.01 M phosphate buffer, pH 7.0, at 37°C. AP-N-catalyzed hydrolysis was studied under the same conditions and an AP-N concentration of 1.6 mU/ml. The cytokines (5–100 μM) were incubated with DP IV (up to 2.6 U/ml) and/or AP-N (up to 4 U/ml) up to 72 h.

The degradation of oligopeptides and cytokines and the generation of dipeptides and amino acids were measured by capillary electrophoresis and HPLC, respectively.

2.3. *Determination of* K_m *and* V_{max}

For determination of K_m and V_{max} six concentrations of each peptide in the range from 25 to 800 μM were incubated with 4.5 mU/ml DP IV in 0.01 M phosphate buffer, pH 7.0, at 37°C. Concentrations of the substrates were measured by capillary electrophoresis. The initial velocity of the hydrolysis reactions were calculated by fitting the data using Excel-Solver (Microsoft) and a first order reaction equation. K_m and V_{max} were calculated by fitting the V/S-characteristic using the Michaelis–Menten equation.

2.4. *Detection of peptides by capillary electrophoresis*

Peptides were analyzed by the method of capillary free zone electrophoresis using the Biofocus 3000 system of Bio-Rad. The standard conditions for separation were: capillary = 25 μm × 24 cm, coated; load = pressure 20 psi × s^{-1}; run = 12 kV with 0.1 M phosphate buffer, pH 2.5; detection at 200 nm.

2.5. *Determination of amino acids by HPLC*

Determination of amino acids generated by AP-N-catalyzed hydrolysis was performed by detection of their *o*-phthaldialdehyde derivatives at 330 nm. After deproteination of the samples, the amino acids were derivatized with *o*-phthaldialdehyde according to the method described in Pharmacia HPLC Application Note 497. For separation by reverse-phase chromatography a RoSil-C18 column (Bio-Rad) and a 30 min phosphate buffer/acetonitril gradient were used.

3. RESULTS

3.1. *Hydrolysis of synthetic peptides with cytokine-like sequences*

The investigated synthetic oligopeptides with sequences analogous to the N-terminal part of natural human cytokines are summerized in Table I.

All of the cytokine analogous oligopeptides studied were found to be hydrolyzed by DP IV, generating the proline-containing dipeptide. The rate of degradation, however, was strongly influenced by the chain length and glycosyl residues. As shown particularly for interleukin-2 partial sequences, DP IV was capable of hydrolysing N-terminal oligopeptides of IL-2 up to a chain length of at least 24 amino acids. Whereas the tetra- and

Table I

Investigated oligopeptides and cytokines

Peptide	Sequence	Number of amino acids	Hydrolysis by DP IV
IL-1β(1–6)	APVRSL	6	+
IL-2(1–4)g	APT(g)S	4	+
IL-2(1–5)g	APT(g)SS	5	+
IL-2(1–6)	APTSSS	6	+
IL-2(1–6)g	APT(g)SSS	6	+
IL-2(1–8)g	APT(g)SSSTK	8	+
IL-2(1–10)g	APT(g)SSSTKKT	10	+
IL-2(1–12)	APTSSSTKKTQL	12	+
IL-2(1–24)	APTSSSTKKTQLQLEHLLLDLQMI	24	+
Met-IL-2(1–6)	MAPTSSS	7	hydrolysis by AP-N and DP IV
Met-IL-2(1–12)	MAPTSSSTKKTQL	13	hydrolysis by AP-N and DP IV
Lymphotoxin (1–5)	LPGVG	5	+
murine IL-6(1–12)	FPTSQVRRGDFT	12	+
murine IL-6(1–12)-POE	FPTSQVRRGDFT-POE	12	+
rG-CSF	MTPLGPASSLP...	174	–
rIL-1α	FSFLSNV...	156	–
rIL-1β	APVRSLNCTL...	153	–
nIL-2	APTSSSTKKT...	133	–
rIL-2	MAPTSSSTKKT...	134	–

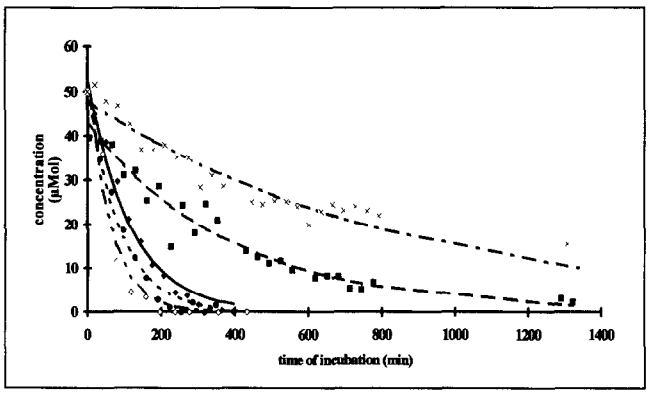

Fig. 1. DP IV hydrolysis of IL-2 oligopeptides with different chain lengths (50 μM). - -●- -, IL-2(1–4)g; – -◇- –, IL-2(1–6); —◆—, IL-2 (1–6)g; – -■- –, IL-2(1–8)g; – -×- –, IL-2(1–10)g. DP IV activity = 2.6 mU/ml.

hexapeptides were totally degraded within 6 h, the decapeptide needed more than 24 h for total hydrolysis by DP IV (2.6 mU/ml) (Fig. 1). IL-2(1–24) peptides were only hydrolyzed by < 10% within 48 h using a substrate concentration of 950 μM and a 1,000-fold higher enzyme concentration (2.6 U/ml, data not shown). A similar effect was observed for murine IL-6(1–12) and the IL-6 dodekapeptide coupled to polyoxyethylene (molecular weight approximately 4,500 Da). Whereas the free dodecapeptide was totally hydrolyzed within 4 h, the polymer-bound dodecapeptide was degraded by only 5% within this time (not documented).

Kinetic data were determined for glycosylated IL-2 oligopeptides and for the non-glycosylated IL-1β and IL-2 hexapeptides (Table II). The V_{max}/K_m values were negatively correlated with increasing molecular weight of the oligopeptides. Comparing glycosylated and non-glycosylated hexapeptides, it could be shown that glycosylation at the Thr^3 has a negative effect on the velocity of hydrolysis of these oligopeptide by DP IV.

A number of recombinant cytokines expressed in bacteria exhibit methionine at their N-terminus. Therefore, we studied the cleavage of a methionine residue from this position by AP-N. Using synthetic hepta- and tridekapeptide with the N-terminal sequence of human rIL-2, we could demonstrate that AP-N is able to split off the N-terminal methionine from these oligopeptides (not shown). No degradation was found with DP IV. Using IL-2(1–12) without methionine in the first position, it could be demonstrated that Ala–Pro is removed totally after 2 h by DP IV. Combined treatment of this oligopeptide by both enzymes leads to total degradation. A concerted action of AP-N and DP IV was also shown in the case of hydrolysis of the murine IL-6(1–12) dodekapeptide (not documented).

3.2. *Treatment of several cytokines by soluble DP IV and AP-N*

The N-terminal sequences of the natural and recombinant cytokines studied with respect to degradation by DP IV and/or AP-N are summerized in the lower part of Table I.

Studying the hydrolysis of these cytokines at concentrations in the range of 10^{-6}–10^{-4} M by DP IV and AP-N alone as well in combination, we found no degradation even at enzyme concentrations up to 1,000-fold higher (maximal 4 U/ml and 2.6 U/ml for AP-N and DP IV, respectively) than in the experiments described above with the oligopeptides. This holds true for data obtained by capillary electrophoresis as well as by HPLC determination of the amino acids.

These results strongly suggest that, in contrast to oligopeptides, intact cytokines such as IL-1α, IL-1β, IL-2 and G-CSF, seem to be resistant to the action of soluble DP IV and AP-N.

4. DISCUSSION

Oligopeptides with the N-terminal sequences of selected cytokines, such as human IL-1β, IL-2, lymphotoxin and murine IL-6, were studied with respect to their degradability by DP IV and AP-N. We could demonstrate that different N-terminal oligopeptides of these cytokines are suitable substrates for the DP IV. The hydrolysis rate of these types of peptides was found to be negatively correlated to their chain length or molecular weight, respectively. This is in line with data of Nausch et al. [27] who studied the DP IV-catalyzed degradation of different biological active peptides, e.g. substance P, β-casomorphine, trypsinogen and monomeric fibrin, as well as intact nIL-2. In contrast to these data, however, Bongers et al. [28] reported that in the case of growth-hormone releasing factor, the hydrolysis rate by DP IV of longer peptides (44 amino acids) is higher than that of shorter peptides (3 and 11 amino acids).

Cytokine-analogous oligopeptides with an additional N-terminal methionine were included in our studies because N-terminal methionine is present in many recombinant cytokines. Our data show that AP-N is able to remove methionine from oligopeptides, and that a con-

Table II

Kinetic constants of DP IV-catalyzed hydrolysis of peptides with cytokine partial sequences

Substrate	Number of amino acids	Molecular weight	K_m (μmol)	V_{max} (μmol/min)	V_{max}/K_m (min^{-1})
IL-2(1–6)	6	548.6	57	3.5	0.061
IL-2(1–4)g	4	581.6	27	1.9	0.070
IL-1β(1–6)	6	641.8	64	2.4	0.037
IL-2(1–5)g	5	668.7	75	1.2	0.016
IL-2(1–6)g	6	755.8	65	1.5	0.023
IL-2(1–8)g	8	985.0	19	0.335	0.018
IL-2(1–10)g	10	1,214.3	199	0.91	0.005

certed action of AP-N and DP IV results in their total degradation.

However, in contrast to oligopeptides with a chain length of up to 24 amino acids, intact cytokines such as rIL-1α, rIL-1β, rIL-2, nIL-2 and G-CSF, were found to be resistant to hydrolysis by purified DP IV and AP-N alone as well as in combination. These data obtained with natural and recombinant cytokines, as well as the results concerning glycosylated peptide sequences of IL-2, are in contradiction to the hypothesis discussed by Nausch [27] which states that glycosylation of natural IL-2 should be the reason for its stability against hydrolysis by DP IV. The most probable explanation for this resistance might be a limited or lack of susceptibility to the N-terminal part of the investigated cytokines for these enzymes under experimental conditions. Analysis of the 3D structures of IL-1α, IL-1β and IL-2, however, demonstrated that the N-terminal part of these cytokines is on the outside of the molecule, i.e. on the surface, and should be accessible to the exopeptidases [22,29]. Obviously additional steric factors influence their susceptibility to soluble exopeptidases.

Our data suggest that hydrolysis of cytokines by cellular exopeptidases needs possibly one or more preceding endoproteolytic steps, and DP IV and AP-N could play a role in modification of the generated N-terminal peptides with the conserved proline in the second or third position. For IL-1β it was shown by Obal [30], Palaszynski [31] and Boraschi [32], that oligopeptides with different internal subsequences of these cytokines had different IL-1β activities. Investigations with N-terminal subsequences are lacking as yet. With regard to preceding endoproteolysis, data published by Bauvois et al. [9] are also interesting; they reported that DP IV is involved in the hydrolysis of an 8 kDa cleavage product of TNF-α generated by the action of other peptidases on a membrane preparation of U937 cells. As an alternative mechanism for influencing the susceptibility of cytokines to exopeptidase-catalyzed processing, conformational changes due to binding of these factors to their specific receptor can not, as yet, be excluded.

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REFERENCES

[1] Hazuda, D.J., Strickler, J., Simon, P. and Young, P.R. (1991) J. Biol. Chem. 266, 7081–7086.
[2] Schmidt, J.A. and Bomford, R. (1991) Cytokine 3, 240–245.
[3] Howard, A.D., Chartrain, N., Ding, G.F., Kostura, M.J., Limjuco, G., Schmidt, J.A. and Tocci, M.J. (1991) Agents Actions Suppl. 35, 77–83.
[4] Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molinaux, S.M., Weidner, J.R., Aunis, J. et al. (1992) Nature 356, 768–74.
[5] Van Damme, J. (1992) in: The Cytokine Handbook (Thomson, A., ed.) pp. 201–214, Academic Press, London.
[6] Clark-Lewis, I.C., Schumacher, C., Baggiolini, M. and Moser, B. (1991) J. Biol. Chem. 266, 23128–23134.
[7] Moser, B., Dewald, B., Barella, L., Schumacher, C., Baggiolin, M. and Clark-Lewis, I. (1993) J. Biol. Chem. 268, 7125–7128.
[8] Bauvois, B., Sancéau, J. and Wietzerbin, J. (1992) Eur. J. Immunol. 22, 923–930.
[9] Wahl, S.M., McCartney-Francis, N. and Mergenhagen, S.E. (1989) Immunol. Today 10, 258–261.
[10] Küllertz, G., Fischer, G., Barth, A. (1986) in: Beiträge zur Wirkstofforschung, Heft 27, Institut für Wirkstofforschung der Akademie der Wissenschaften der DDR und Institut für Pharmakologische Forschung der Pharmazeutischen Industrie, Berlin.
[11] Hanson, H., Hütter, H.J., Mannsfeldt, H.-G., Kretschmer, K. and Sohr, C. (1967) Hoppe Seylers Z. Physiol. Chem. 348, 680–688.
[12] Schön, E., Mansfeld, H.-W., Demuth, H.U., Barth, A. and Ansorge, S. (1985) Biomed. Biochem. Acta 44, K9–K15.
[13] Ansorge, S. and Schön, E. (1987) in: Advances in the Biosciences, vol. 65 (Showen, R.L. and Barth, A., eds.) pp. 3–10, Pergamon Press, Oxford.
[14] Ansorge, S., Schön, E. and Kunz, D. (1991) Biomed. Biochim. Acta 50, 799–807.
[15] Schön, E., Eichmann, E., Jahn, S., Kopp, J., Volk, H.-D. and Ansorge, S. (1988) Biol. Zentralbl. 107, 141–149.
[16] Schön, E. and Ansorge, S. (1990) in: Dipeptidyl peptidase IV: General and Applied Aspects (Barth, A., Schowen, R.L., eds.) Beiträge zur Wirkstofforschung Heft 38, pp. 84–96, Institut für Wirkstofforschung der Akademie der Wissenschaften der DDR und Institut für Pharmakologische Forschung Berlin GmbH, Berlin.
[17] Schön, E., Born, I., Demuth, H.-U., Faust, J., Neubert, K., Steinmetzer, T., Barth, A. and Ansorge, S. (1991) Biol. Chem. Hoppe Seyler 372, 305–311.
[18] Scholz, W., Feller, A.C., Ulmer, A.J. and Flad, H.-D. (1986) in: Leukocyte Typing II, Vol. 1, Human T lymphocytes, (Reinherz, E.L., Haynes, B.F., Nadelere, L.M. and Bernstein, I.D., eds.) pp. 489–501, Springer, New York.
[19] Mattern, T., Scholz, W., Feller, A.C., Flad, H.D. and Ulmer, A.J. (1991) Scand. J. Immunol. 33, 737–748.
[20] Kubota, T., Flentke, G.R., Bachovchin, W.W. and Stollar, B.D. (1992) Clin. Exp. Immunol. 89, 192–197.
[21] Yanofsky, S.D. and Zurawski, G., (1990) J. Biol. Chem. 265, 13000–6.
[22] Veerapandian, B., Gilliland, G.L., Raag, R., Svensson, A.L., Masui, Y., Hirai, Y. and Poulos, T.L. (1992) Proteins 12, 10–23.
[23] Ju, G., Collins, L., Kaffka, K.L., Tsien, W.-H., Chizzonite, R., Crowl, R., Bhatt, R. and Kilian, P.L. (1987) J. Biol. Chem. 262, 5723–5731.
[24] Lillquist, J.S., Simon, P.L., Summers, M., Jonak, Z. and Young, P.R. (1988) J. Immunol. 141, 1975–1981.
[25] Mosley, B., Dower, S.K., Gillis, S. and Cosman, D. (1987) Proc. Natl. Acad. Sci. USA 84, 4572–4576.
[26] Rahfeld, J., Schutkowski, M., Faust, J., Neubert, K., Barth, A. and Heins, J. (1991) Biol. Chem. Hoppe Seyler 372, 313–318.
[27] Nausch, I., Mentlein, R. and Heymann, E. (1990) Biol. Chem. Hoppe Seyler 371, 1113–1118.
[28] Bongers, J., Lambros, T., Ahmad, M. and Heimer, E. (1992) Biochim. Biophys. Acta 1122, 147–153.
[29] Brandhuber, B.J., Boone, T., Kenney, W.C. and McKay, D.B. (1987) Science 238, 1707–1709.
[30] Obal, J.F., Opp, M., Cady, A.B., Johannsen, L., Postlethwaite, A.E., Poppleton, H.M., Seyer, J.M. and Krueger, J.M. (1990) Am. J. Physiol. 259, 345–442.
[31] Palaszynski, E.W. (1987) Biochem. Biophys. Res. Commun. 147, 537–542.
[32] Boraschi, D., Antoni, G., Perni, F., Villa, L., Nencioni, L., Ghiara, P., Presentini, R. and Tagliabue, A. (1990) Eur. Cytokine 1, 21–26.