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Fluorescent Cytochemical Detection of Dipeptidyl Peptidase IV and Tripeptidyl Peptidase I by Novel Substrates

M. Dimitrova, I. Ivanov**, A. Dikov**

**Institute of Experimental Morphology and Anthropology, Bulgarian Academy of Sciences*
***Institute of Experimental Pathology and Parasitology, Bulgarian Academy of Sciences, Sofia*

New fluorogenic substrates for dipeptidyl peptidase IV and tripeptidyl peptidase I, based on 2-anthraquinonylhydrazine, are synthesized and used for the cytochemical detection of those peptidases in cultured avian cells and rat peritoneal macrophages. The new fluorescent procedure includes coupling reaction between 2-anthraquinonylhydrazine, released on enzyme action and 3-nitrobenzaldehyde. The hydrazone, obtained on this reaction, precipitates on the enzyme locations and marks them by bright orange-red fluorescence, when excited in the green area of the visible spectrum. The method is very sensitive and permits the visualization of the enzymes at the lack of any fluorescent background noise.

Key words: Dipeptidyl petidase IV, Tripeptidyl peptidase I, fluorescent method, enzyme cytochemistry.

Introduction

Dipeptidylpeptidase IV (DPP IV; EC 3.4.14.5), a membrane-bound peptidase, cleaves preferably Glycyl-Proline dipeptides from the NH_2-terminal of oligopeptides at optimal pH 7.8. It is widely distributed in animal organs and tissues [5, 7] and is discovered to be identical to T cell activation marker CD 26 [14]. DPP IV is present also in the lysosomes of T lymphocytes and cultivated cells [10].

Tripeptidyl peptidase I (TPP I; EC 3.4.14.9) is a lysosomal enzyme, cleaving tripeptides from the NH_2-terminal of oligopeptides at optimal pH 4.5 [1]. It is found in many animal and human organs and in tumor cells [1]. TPP I can hydrolyze synthetic substrates with Gly-Pro-Met amino acid sequence. Since this triad is typical for collagen fibrils, TPP I is believed to be crucial for the degradation of collagen [12].

Recently, we introduced a novel chromogenic method for the visualization of peptidases, which we applied both for DPP IV and TPP I [4, 9]. The substrates were based on 1-anthraquinonylhydrazine (1-AH) and aromatic aldehydes were used as coupling agents. The only fluorescent method for peptidases, proposed by now, is used solely for the detection of DPP IV, but not for TPP I. It employs the substrate Gly-Pro-4-methoxynaphthylamide (Gly-Pro-MNA) and 5-nitrosalicylaldehyde (NSA) as auxiliary reagent. However, this technique suffers a lot of drawbacks [13].

In the present paper we describe the synthesis of two fluorogenic substrates – Gly-L-Pro-2-anthraquinonylhydrazide (Gly-Pro-2-AH) for DPP IV and Gly-L-Pro-L-Met-2-anthraquinonylhydrazide (Gly-Pro-Met-2-AH) for TPP I and their application for the fluorescent visualization of the enzymes in cells of different origin.

Material and Methods

Synthesis of the substrates. 2-Aminoanthraquinone (Merck) was sublimated at 300°C in vacuo. 2-AH was synthesized from the so purified 2-aminoanthraquinone by diazotization with isoamylnitrite in acetic acid and sulfuric acid [8] and a subsequent reduction of the diazonium sulfate to hydrazine by sodium sulfite [11]. *DPP IV substrate*: Z-Gly-L-Pro-OH (protected amino acids were from Bachem) was coupled to 2-AH using the dicyclohexylcarbodiimide method [2]. Z-protection was cleaved with HBr/acetic acid to give the substrate Gly-Pro-2-AH as HBr-salt. *TPP I substrate:* Boc-Met was coupled to 2-AH by the dicyclohexylcarbodiimide method [2]. The Boc-protection was cleaved with 6N HCl in dioxan in the presence of m-cresol. The obtained compound was coupled with Boc-Gly-L-Pro-OH by the same method [2], using N-ethylmorpholine as HCl-acceptor. The Boc-protection was cleaved as above and the substrate Gly-Pro-Met-2-AH was obtained as a HCl-salt.

Cells and incubation media. Cell cultures (200th passage) from the permanent cell line LSTC-SF1 (Mc 31) were used. The cell line was established from turkey embryo cells, transformed by the avian myelocytomatosis virus strain Mc-31 [15, 16]. The cell cultures were grown in DMEM/F_{12} mixture (1:1), supplemented with 5 % fetal calf serum in Petri dishes (60 mm) on cover slides. After 3 days of cultivation, the cells were washed in isotonic solution and used for cytochemical investigations.

Three months old Wistar rats were injected intraperitoneally with 0,5 ml heterologous serum. On the 4th day, they were killed and peritoneal lavage with 199 medium was carried out. Cells, collected by centrifugation were re-suspended in homologous serum and smears were made on slides, covered by chrome-alum jelly.

All the cell preparations were fixed in paraformaldehyde vapors at room temperature for 2 min. Then, they were fixed in acetone/chloroform (1:1) for 5 min at –20°C. Incubation solution for DPP IV consisted of 0.5 mM substrate (Gly-Pro-2-AH) in 0.05 M borate buffer. The incubation lasted an hour. After that, the preparations were transferred in 0.2 M acetate buffer, pH 4.5, supplied with 5 mM 3-nitrobenzaldehyde (NBA) and were kept there for 3 hours. For the visualization of TPP I the cells were incubated for an hour in a solution, containing 0.5 mM substrate (Gly-Pro-Met-2-AH) and 3 mM NBA in 0.1 M acetate buffer, pH 4.5. After a post-fixation in 4 % formaldehyde in 0.1 M cacodilate buffer, pH 7.4, all the preparations were embedded in 70 % saccharose and 10 % glycerol, dissolved in 0.01 M sodium hydroxide. They were examined under a fluorescent microscope OPTON IM 35 using a filter combination G 546, FT 580, LP 590. Microphotographs were made on a black & white NEOPAN 400 Professional Fujifilm.

Controls were treated in the same manner, but heated to 80°C for 10 min in water before the incubation. Other controls were incubated only in buffered NBA.

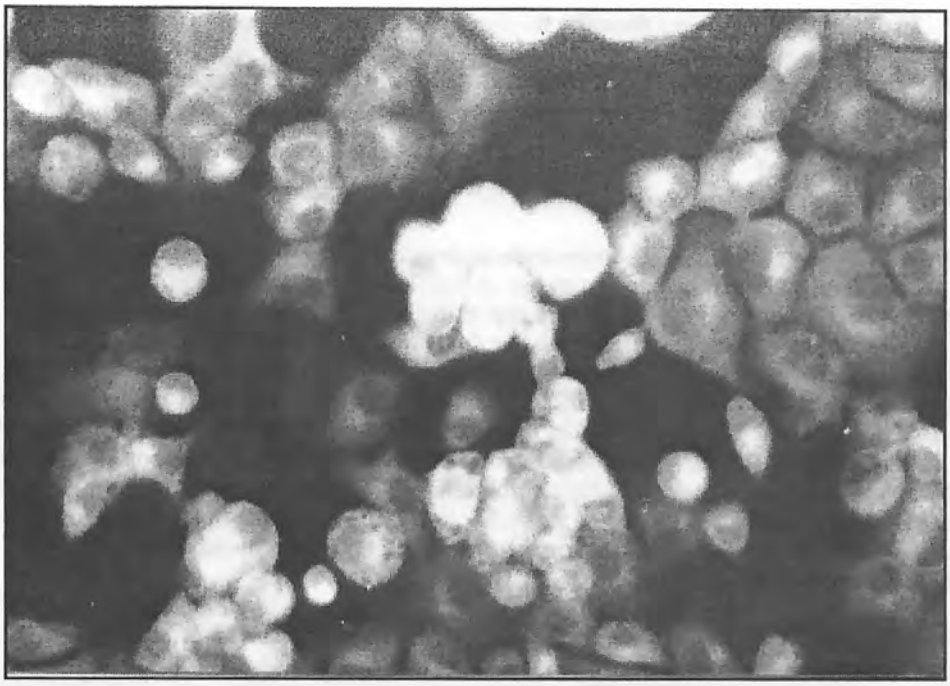

Fig. 1. TPP I in the cell line LSTC-SF1 (Mc31). Very strong reaction in the cytoplasm of the cells (Microphoto × 500)

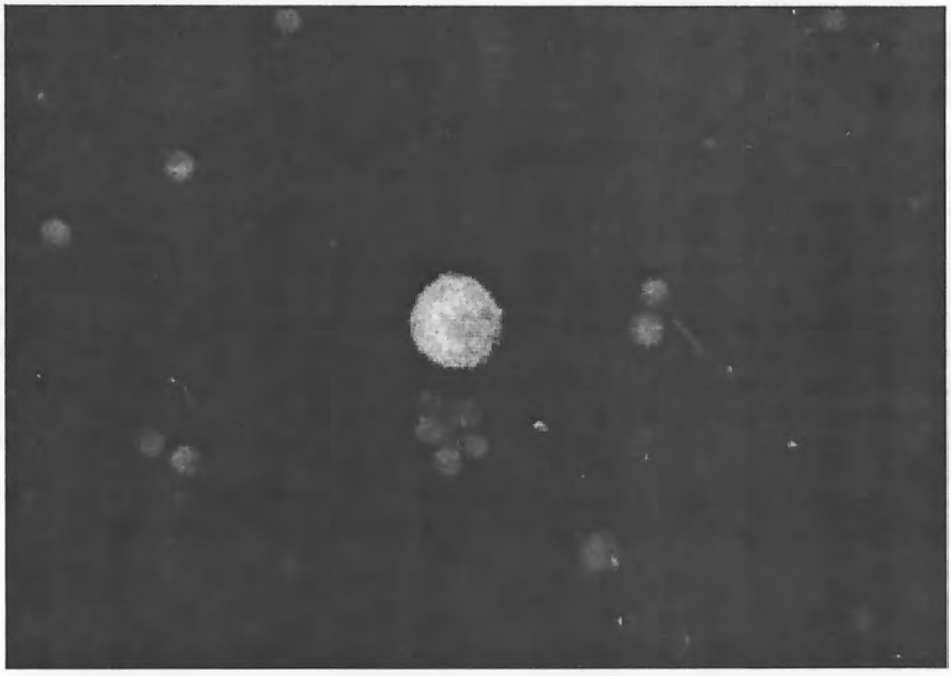

Fig. 2. TPP I in rat peritoneal cells. Numerous fine hydrazone granules fill the cytoplasm of mastocytes, whereas the reaction in the macrophages is much weaker (Microphoto × 500)

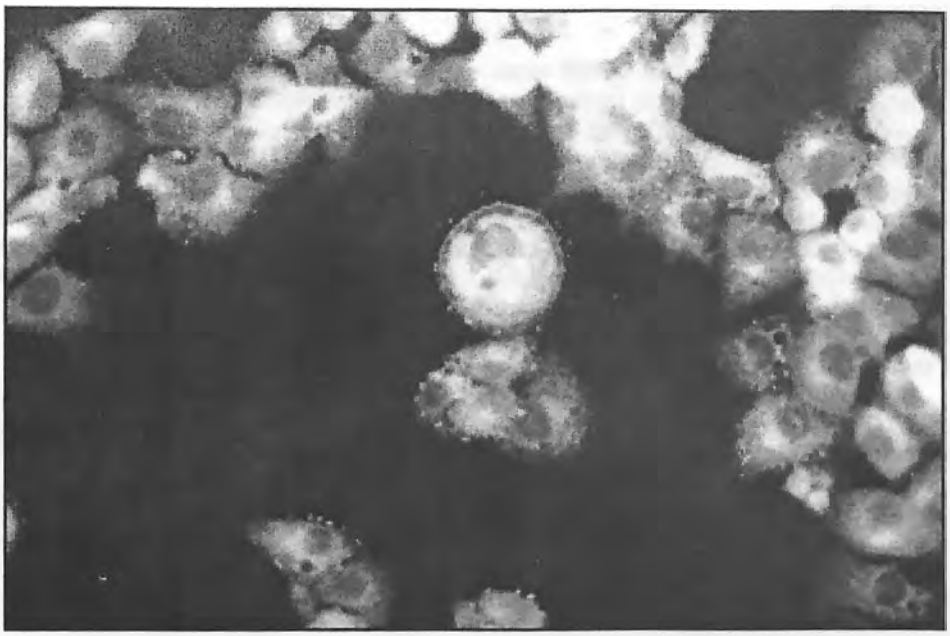

Fig. 3. DPP IV in the cell line LSTC-SF1 (Mc-31). Granular reaction within the cytoplasm of the cells (Microphoto × 500)

Results

Both types of controls were free of any non-specific staining.

The newly synthesized substrates were hydrolyzed quickly by the enzymes to release 2-AH, which is practically insoluble in aqueous media and precipitated on the sites of enzyme activity. Yet, 2-AH fluoresces very weakly. So, to obtain a highly fluorescent product, it had to be converted into 3-nitrobenzylidene-2-anthraquinonyl hydrazone by the use of NBA. This hydrazone shows a very intensive orange-red fluorescence on excitation by green light (λ = 520-580 nm). In the case of TPP I the aldehyde was supplied in the incubation medium, where it reacted simultaneously with 2-AH. This was possible, because TPP I has an acid pH optimum, at which the coupling reaction is performable. In the case of DPP IV, which is active in alkaline solutions, this second reaction step had to be carried out in acetic buffer, pH 4.5, supplied with NBA. In both cases the enzyme activity was revealed at minimal or none fluorescent background noise. TPP I was visualized as numerous fine granules within the cultured cells line LSTC-SF1 (Mc-31) (Fig. 1). Very strong reaction for the same enzyme was visible in the rat peritoneal mastocytes (Fig. 2), whereas the reaction in rat macrophages was much weaker. This is in accordance with previous findings, revealing that mastocytes lysosomes are rich of proteases [3]. DPP IV was also seen within the cytoplasm of the cells from line LSTC-SF1 (Mc31) (Fig. 3). Such granulated intracellular reaction for DPP IV has been reported and before [10].

Discussion

Fluorescent methods are 200 to 400 times more sensitive than the chromogenic [13]. The only available fluorescent method for peptidases uses substrates, possessing MNA as a leaving group. MNA, released on the enzyme hydrolysis, couples with NSA to give a fluorescent Shiff's base, which marks the enzyme locations. However, this procedure has a lot of obstacles. MNA is soluble in water and diffuses from the places of its origin. The coupling rate between it and NSA is high only in acid medium, so that the method cannot be applied for alkaline peptidases [6]. The Shiff's base is rough crystalline, fluoresces in yellow when excited by blue light, so that the tissue autofluorescence results in a substantial background noise. Also, it decomposes upon excitation and the fluorescence in the preparations quickly reduces. Thus, no convenient fluorescent cytochemical method for peptidases is available yet.

In previous papers [4, 9] we introduced Gly-Pro-1-AH and Gly-Pro-Met-1-AH as substrates for the chromogenic localization of DPP IV and TPP I. The enzyme activity liberates 1-AH, which reacts with an aromatic aldehyde in the incubation medium (TPP I) or in a post-incubation solution (DPP IV) to give deeply colored hydrazone. However, neither 1-AH, nor its hydrazones do fluoresce. So, this method, though having many advantages, is chromogenic. It came into view, that 2-AH (in contrast to 1-AH) has a very low fluorescence, which amplifies greatly upon its conversion to a hydrazone with NBA. The newly synthesized substrates for DPP IV and TPP I are very quickly hydrolyzed by the enzymes and the practically insoluble in water and buffers 2-AH precipitates on the enzyme sites. Further on, it was converted to hydrazone by treatment with NBA. The obtained hydrazone is amorphous and has a very stable and intensive orange-red fluorescence, which does not decrease upon excitation. Another advantage of this technique is that the hydrazone is to be excited by green light, an area in which the cell fluorochromes are not excited. Thus, the autofluorescence of the cells is invisible and the enzymes are visualized at the lack of background noise. Using the newly developed technique we visualized fluorescently DPP IV and TPP I in two types of cells.

Since the newly developed method promises to be very sutable for the detection of peptidases, the synthesis of other peptidases substrates based on 2-AH is now in progress.

References

1. Andersen, K.-J., J. K. McDonald. Subcellular distribution of renal tripeptide-releasing exopeptidases active on collagen-like sequences. – Am. J. Physiol., **F252,** 1987, 890-898.
2. Bodanszky, M., A. Bodanszky. The dicyclohexylcarbodiimide method. – In: The practice of peptide synthesis. Berlin, Academie-Verlag 1985, 143.
3. Caughey, G. H., N. F. Viro, L. D. Calonico, D. M. McDonald, S. C. Lazarus, W. M. Gold. Chymase and Tryptase in dog mastocytoma cells: asynchronous expression as revealed by enzyme cytochemical staining. – J. Histochem. Cytochem., **36,** 1988, 1053-1060.
4. Dikov, A., M. Dimitrova, I. Ivanov, R. Krieg, K.-J. Halbhuber. Original method for the histochemical demonstration of tripeptidyl aminopeptidase I. – Cell. Mol. Biol., **46,** 2000, 1219-1226.
5. Gossrau, R. Peptidase II. Zur Lokalisation der Dipeptidylpeptidase IV (DPP IV). Histochemische und biochemische Untersuchung. – Histochemistry, **60,** 1979, 231–248.
6. Gossrau, R. Fluorescence histochemical detection of hydrolases in tissue sections and culture cells. – Histochemistry, **79,** 1983, 87-94.
7. Hartel, S., R. Gossrau, C. Hanski, W. Reutter. Dipeptidylpeptidase IV (DPP IV) in rat organs. Comparison of immunohistochemistry and activity histochemistry. – Histochemistry, **89,** 1988, 151-161.
8. Kaufler, F. Ueber Azo- and Azomethinderivate des β–Aminoanthrachinons. – Ztschr.f.Farbenchemie, **2,** 1903, 469-473.

9. Krieg, R., M. Dimitrova, A. Dikov. Glycyl-L-Prolyl-1-anthraquinonylhydrazide – a new substrate for the histochemical detection of dipeptidyl peptidase IV. – Acta Morph. Anthr., **8,** 2003, 63-68.
10. Lojda, Z. Studies on glycyl-proline naphthylamidase. I. Lymphocytes. – Histochemistry, 54, 1977, 299-309.
11. Moehlau, R. Ueber eine neue Synthese der Anthrachinonyl-hydrazin. – Chem.Ber., **45,** 1912, 2244-2247.
12. Page, A. E., K. Fuller, T. J. Chambers, M. J. Warburton. Purification and characterization of a tripeptidyl peptidase I from human osteoclastomas: evidence for its role in bone resorption. – Arch. Biochem. Biophys., **306,** 1993, 354-359.
13. Raap, A. K. Localization properties of fluorescent cytochemicalenzyme procedures. – - Histochemistry, **84,** 1986, 317-321.
14. Ulmer, A. J., T. Mattern, A. C. Feller, E. Heymann, H.-D. Flad. CD 26 antigen is a surface dipeptidyl peptidase IV (DPP IV) as characterized by monoclonal antibodies clone TII-19-4-7 and 4EL1-C7. – Scand. J. Immunol., **31,** 1990, 429-443.
15. Иванов, И., Т. Тодоров,С. Недялков. Стабилна линия от ембрионални клетки на пуйка, трансформирани от миелоцитоматозния вирусен щам Мс-31. – Обща сравн. патол., **15,** 1984, 44-52.
16. Иванов, И. Стабилни линии от клетки, трансформирани от миелоцито-матозния вирусен щам Мс-31. – Обща Сравн. Патол., **28,** 1991, 19–30.