JOURNAL OF PROTEOMICS 75 (2012) 1472–1485

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/jprot

Proteinase 3 carries small unusual carbohydrates and associates with αlpha-defensins

Morten Zoega[a], *Tina Ravnsborg*[b], *Peter Højrup*[b,*], *Gunnar Houen*[a,b], *Christian Schou*[a,1]

[a]*Department of Clinical Biochemistry and Immunology, Statens Serum Institut, DK-2300 Copenhagen S., Denmark*
[b]*Institute of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense M, Denmark*

ARTICLE INFO

Article history:
Received 19 August 2011
Accepted 15 November 2011
Available online 30 November 2011

Keywords:
Proteinase 3
Glycosylation
α-defensin associations
α1-antitrypsin inhibitor
Neutrophil proteins
Mass spectrometry

ABSTRACT

The neutrophil granulocyte is an important first line of defense against intruding pathogens and it contains a range of granules armed with antibacterial peptides and proteins. Proteinase 3 (PR3) is one among several serine proteases of the azurophilic granules in neutrophil granulocytes. Here, we characterize the glycosylation of PR3 and its association with antimicrobial human neutrophil peptides (HNPs, α-defensins) and the effect of these on the mechanism of inhibition of the major plasma inhibitor of PR3, α1-antitrypsin. The glycosylation of purified, mature PR3 showed some heterogeneity with carbohydrates at Asn 102 and 147 carrying unusual small moieties indicating heavy processing. Mass spectrometric analysis and immuno blotting revealed strong association of highly purified PR3 with α-defensins and oligomers hereof. Irreversible inhibition of PR3 by α1-antitrypsin did not affect its association with defensins. Other proteins from neutrophil granules were also found to be associated with defensins, whereas purified plasma proteins did not carry defensins. These results point to a role of defensins in controlling and targeting the activity of neutrophil granule proteins.

1. Introduction

The neutrophil granulocyte is an important first line of defense against intruding pathogens. With up to 70% by numbers, the human neutrophils are the largest constituent of the human white blood cells. The neutrophils contain large amounts of cytoplasmic granules with specific purposes and protein content tailored by the biogenesis at different steps along the myeloid differentiation and maturation [1,2].

In contrast to previous beliefs, it is now recognized that neutrophil granules develop continuously and through heterogeneous subsets with individual granule protein contents

Abbreviations: PR3, Proteinase 3; NHP, human neutrophil peptide; WG, Wegener's granulomatosis; PMN, polymorphonuclear leukocytes; PAR-2, protease-activated receptor-2; IFN-γ, interferon gamma; ANCA, anti-neutrophil cytoplasmic antibodies; MPO, myeloperoxidase; SAP, serum amyloid P; mAb, monoclonal antibodies; pAb, polyclonal antibodies; PMSF, phenylmethylsulfonyl fluoride; HSA, human serum albumin; TTR, transthyretin; BCIP/NBT, 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium; EDTA, Ethylene diamine tetra aceticacid; FBP, folate binding protein; β2-GP1, β2-glycoprotein 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; PNGase F, N-glycosidase F; RT, room temperature; MeCN, acetonitrile; AspN, Endoproteinase AspN; TFA, trifluoroacetic acid; FA, formic acid; CID, collision induced dissociation; MS, mass spectrometry; MALDI, Matrix-assisted laser desorption-ionization; TTN, Tris-Tween-Sodium chloride.

* *Corresponding author*. Tel.: +45 60112371; fax: +45 65502467.
E-mail address: php@bmb.sdu.dk (P. Højrup).
[1] Present address: Novozymes A/S, DK-2880 Bagsværd, Denmark.

1874-3919/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jprot.2011.11.019

[1,3,4]. The biogenesis of the granules is regulated by transcription factors differentiating the expression profile over time [5].

Proteinase 3 (PR3) is a serine protease and a constituent of the peroxidase positive subset of the azurophil granules characterized by the presence of myeloperoxidase (MPO), together with several other proteases and antibacterial proteins [1,6-8].

The main constituent of the neutrophil granules is the defensins, which belong to the family of human antimicrobial peptides [4,9]. The defensins exert a broad spectrum of antimicrobial activities [10-16] as well as associations with other molecules [17-20]. Three members of the α-defensin family (HNP 1–3) are structurally almost identical with an approximate molecular weight of 3.5 kDa and an overall tightly packed spherical structure and net positive charge due to three disulfide bonds and a high content of arginine amino acid residues. The three α-defensins share the conserved peptide sequence of α-defensin 2, although the sequences of α-defensin 1 and 3 are extended N-terminally by Alanine and Aspartic acid residues respectively [14,15]. The human neutrophil contains large amounts of defensins, the three α-defensins 1–3 comprising up to 7% of the total cell protein pool [21], and 97% of the defensin pool, mainly localized to the azurophilic subset where they approximate 50% of the total protein content at 50 mg/ml concentration [22]. Upon activation, the neutrophils release as much as 10% of these cytotoxic defensin peptides to the environment [23]. Such a large release calls for tight regulation and the involvement of several human plasma proteins has been implicated in the regulation of the defensin activity [17,18,24].

PR3 has been implicated with a number of human diseases. In Wegener's granulomatosis (WG) [25-29] much evidence points to an initial infection trigger and a subsequent evolution into an autoimmune condition characterized by the presence of autoantibodies to neutrophil granulocyte antigens with PR3 as the dominating specificity [30-34].

The localization to the azurophilic granules and the cell surface of polymorphonuclear leukocytes (PMN) [35] hints at a strong participation of PR3 in the local inflammatory response primarily via proteolytic involvement but also through less well understood binding interactions. The proteolytic cell activation through cleavage of protease-activated receptor-2 (PAR-2) modulates maturation of dendritic cells and induces enhanced IFN-γ response in CD4+ T cells of WG patients in vitro [36-38] and modulates inflammatory reactions by cleavage and inactivation of C1 inhibitor [39]. Likewise, binding to PR3 elevates the biological activity of the cell associated pro-inflammatory cytokine IL-32 [40,41]. The proteolytic involvement of the neutrophilic serine proteases in localized immunological and inflammatory events has led to investigations of the natural inhibitory regulation of these proteases by members of the serpin inhibitor family. The serpins constitute the predominant family of primarily serine proteinase inhibitors, where α1-antitrypsin constitutes the prime physiological inhibitor of PR3 [42-45]. Under non-inflammatory conditions the activity of PR3 is tightly regulated by a large surplus of circulating α1-antitrypsin inhibitor. Even though the complex of PR3/α1-antitrypsin is irreversible, several reports point at disturbances of this regulatory inhibition. Some reports suggest that ANCA interfere with both the proteolytic activity of PR3 [46] and clearance of PR3 by α1-antitrypsin inhibition in WG [47,48]. Altogether, the structure of PR3 and its association with other neutrophil granule constituents have important consequences for its biological activities.

In this paper we characterize the glycosylation of PR3 purified from neutrophil granulocytes and reveal a novel association of PR3 with granulocyte α-defensins.

2. Materials and methods

2.1. Reagents

Mouse monoclonal immunoglobulins against PR3 (4A3 (HYB 172-04/IgG2a), 4A5 (HYB 172-05/IgG2a), and 6A6 (HYB 172-01/IgG1)), serum amyloid P (SAP) (HYB 281-05/IgG1), myeloperoxidase (MPO) (clone 2B11/IgG2a), and rabbit PR3 antiserum were produced and tested in-house [49,50]. Alkaline phosphatase-conjugated goat anti mouse IgG and goat anti rabbit IgG antibodies were from Sigma (St.Louis, MI, USA). For detection of non-specific binding and as negative controls the following antibodies were used: rabbit anti-human lactoferrin (ab15811), rabbit anti-human lysozyme (ab34799), rabbit anti cathepsin G (ab20725), rabbit anti azurocidin (ab48132), rabbit anti elastase (ab21595), chicken anti α1-antitrypsin (ab14226), elastase mAb (ab41179), cathepsin G mAb (ab50845), lysozyme mAb (ab36362), α1 antitrypsin mAb (ab9400), α defensins mAb (D1F4, ab64763) and β defensin 1 mAb (ab14425) (Abcam, Cambridge, UK), and α 1–3 human defensin mAb (T1034) (Bachem, Weil am Rhein, Germany). Other reagents were from the following suppliers, N-glycosidase F (Roche, Basel, Switzerland), human α-defensins (HNP 1–3) (HyCult Biotechnology, PB Uden, The Netherlands), PR3 (Wieslab, Lund, Sweden), human α1-antitrypsin, endoproteinase AspN, D-glucose monohydrate (Calbiochem, Merck, Germany), elastase, lysozyme, cathepsin G, azurocidin, MPO (Arotec Diagnostics Ltd., Petone, Wellington, New Zealand), PMSF (Roche, Basel, Switzerland), MeOSuc-Ala-Ala-Pro-Val-OH, MeOSuc-Ala-Ala-Pro-Val-CMK, MeOSuc-Ala-Ala-Pro-Val-pNA (Bachem, Weil am Rhein, Germany), lactoferrin, human serum albumin (HSA), transthyretin (TTR), ovalbumin, (3-Chloro-7-OH)-4-methylcoumarin, BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) substrate tablets, D-mannose, L-fucose, EDTA (Sigma, St.Louis, MI, USA), folate binding protein (FBP), SAP, calreticulin, β2-glycoprotein 1 (β2GP1) (SSI, Copenhagen, Denmark).

2.2. Proteinase 3 purification

PR3 was purified from azurophilic granules isolated from human neutrophil granulocytes as described previously [50,51]. The purity of PR3 during the purification process and in the final product was evaluated by SDS-PAGE in combination with silver staining and mass spectrometry. Furthermore, western blotting and ELISA were performed for cross reactivity analysis with antibodies against other human neutrophil granule proteins.

2.3. SDS-PAGE

SDS-PAGE was performed in the XCell Surelock TM mini-cell using 4–20% Tris–glycine gels (Invitrogen, Carlsbad, CA, USA). Low range pre-stained SDS-PAGE standards were from BioRad