Human Neutrophils Secrete Bioactive Paucimannosidic Proteins from Azurophilic Granules into Pathogen-Infected Sputum*

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Background: Protein paucimannosylation is considered an important invertebrate- and plant-specific glycoepitope.

Results: Azurophilic granule-specific human neutrophil proteins from pathogen-infected sputum displayed significant core-fucosylated paucimannosylation generated by maturation- and granule-specific β-hexosaminidase A and were preferentially secreted from non-lysosomal origins into sputum upon P. aeruginosa stimulation.

Conclusion: Human neutrophils produce, store, and selectively secrete bioactive paucimannosidic proteins.

Significance: This work will aid in understanding the function(s) of human paucimannosylation in glycoimmunology.

Unlike plants and invertebrates, mammals reportedly lack proteins displaying asparagine (N)-linked paucimannosylation (mannose1 → fucose0 → N-acetylglycosamine1Asn). Enabled by technology advancements in system-wide biomolecular characterization, we document that protein paucimannosylation is a significant host-derived molecular signature of neutrophil-rich sputum from pathogen-infected human lungs and is negligible in pathogen-free sputum. Five types of paucimannosidic N-glycans were carried by compartment-specific and inflammation-associated proteins of the azurophilic granules of human neutrophils including myeloperoxidase (MPO), azurocidin, and neutrophil elastase. The timely expressed human azurophilic granule-resident β-hexosaminidase A displayed the capacity to generate paucimannosidic N-glycans by trimming hybrid/complex type N-glycan intermediates with relative broad substrate specificity. Paucimannosidic N-glycopeptides showed significant co-localization with β-hexosaminidase A and the azurophilic marker MPO in human neutrophils using immunocytochemistry. Furthermore, promyelocyte stage-specific expression of genes coding for paucimannosidic proteins and biosynthetic enzymes indicated a novel spatio-temporal biosynthetic route in early neutrophil maturation. The absence of bacterial exoglycosidase activities and paucimannosidic N-glycans excluded exogenous origins of paucimannosylation. Paucimannosidic proteins from isolated and sputum neutrophils were preferentially secreted upon inoculation with virulent Pseudomonas aeruginosa. Finally, paucimannosidic proteins displayed affinities to mannose-binding lectin, suggesting immune-related functions of paucimannosylation in activated human neutrophils. In conclusion, we are the first to document that human neutrophils produce, store and, upon activation, selectively secrete bioactive paucimannosidic proteins into sputum of lungs undergoing pathogen-based inflammation.

Asparagine (N)-linked glycosylation adds unprecedented structural and functional heterogeneity to polypeptide chains by the covalent attachment of oligosaccharides (hereafter called glycans) to motif-specific asparagine residues. Although advances in glycobiochemistry and analytical glycoscience continually improve the understanding of protein N-glycosylation, the structure-function relationships of most protein glycoforms remain unknown. Dysregulation of protein N-glycosylation by the deletion or modulation of its diverse intracellular and extracellular functions is a common cause and/or effect of numerous pathologies (1, 2). Our understanding of the conserved mammalian N-glycosylation as a structural and functional modulator of proteins is most critically built on the principles of the well described glycoprotein biosynthetic machinery (3). The defined complex and dynamic enzymatic biosynthesis of glycoproteins in the secretory pathway produces three well recognized N-glycan classes that are abundantly displayed on mammalian proteins, i.e. high mannose, hybrid, and complex type, all of which are based on a common trimannosylated chitobiose core (2).

Contrary to this dogma, several recent glycomics-based studies indicate that a fourth type of protein N-glycosylation, referred to as paucimannosylation, with monosaccharide compositions less than or equal to the N-glycan trimannosylchitobiose core, i.e. mannose(Mann)1 → fucose(Fuc)0 → N-acetylglycosamine(GlcNAc)2, is present in mammals. These structures do not correspond to the defined N-glycan types nor can their synthesis be described by established mammalian biosynthetic pathways. To date, mammalian protein paucimannosylation has been suggested to be present in (i) human buccal epithelial cells (4), (ii) human colorectal cancer epithelial cells and tissue (5–7), (iii) kidney tissue from mice suffering from systemic
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lupus erythematosus (8), (iv) mouse embryonic neural stem cells (9), and (v) rat brain (10). In addition, we recently indicated the presence of paucimannosylation in pathogen-infected sputum derived from individuals with cystic fibrosis (CF) and upper respiratory tract infection (URTI) (11). Importantly, these observations were all based on molecular profiling of N-glycans released from cell/tissue-derived proteins and thus disregarded the protein carrier identities. Consequently, exogenous origin(s) of paucimannosylation could not be ruled out. Mammalian paucimannosylation was supported by immunohistochemistry and immunocytochemistry of selected human and murine tissues and cells using paucimannose-reactive antibodies (12–14). In general, however, mammals including human and mice have usually been reported to lack protein paucimannosylation (15–21). As such, human paucimannosylation remains controversial in the context of our current understanding of mammalian glycobiology.

Outside the vertebrate subphylum, invertebrates such as Caenorhabditis elegans (16, 18, 22–24) and Drosophila melanogaster (15, 17, 25, 26), plants (27), and other “lower” organisms (28) abundantly produce protein paucimannosylation. Paucimannose synthesis in these species is facilitated by high β-N-acetylhexosaminidase activity, allowing partial suppression of the complex N-glycan biosynthetic route. Although the exact functions and effector mechanisms still remain elusive, the N-glycosylation of the paucimannose-rich C. elegans and D. melanogaster has been associated with roles in the immune response against bacterial pathogens (19) and the organism lifespan (21).

Here, we present unequivocal evidence that paucimannosylation is also a significant host-derived molecular signature of sputum proteins from pathogen-infected human lungs. Enabled by recent developments in system-wide biomolecular detection, we document that inflammation-associated proteins, localizing to the azurophilic granules of human neutrophils, abundantly display paucimannosylation. In line with their presence in specific micro-environments that are central to inflammation and pathogen infection, we confirm that the timely expressed human azurophilic granule-resident β-hexosaminidase A (Hex A) enzymatically facilitates the generation of protein paucimannosylation by trimming hybrid/complex type N-glycan intermediates using a machinery, which is formed during early myeloid maturation, and functionally associate paucimannosidic proteins with roles in innate immunity upon secretion from activated human neutrophils.

**EXPERIMENTAL PROCEDURES**

*Sputum and Bacteria Origin/Handling—Saliva-free whole sputum (>1 ml/donor) was sampled with informed consent from individuals with (n = 5) or without (n = 4) CF by non-invasive expectoration at Westmead Hospital, Sydney, Australia (see Ref. 11 for donor data). Two of the non-CF individuals were diagnosed with URTI and two were diagnosed with pathogen-free pneumonia or chronic obstructive pulmonary disease. The sputum of the seven pathogen-positive individuals was infected primarily by mucoid/non-mucoid Pseudomonas aeruginosa, but also Aspergillus fumigatus, Staphylococcus aureus, and Streptococcus pneumoniae were identified. P. aeruginosa laboratory wound (PAO1) and CF sputum (PASS1–4) strains were isolated and cultured (Table 1). Sputum from all donors showed inflammation characteristics (>1 × 10^6 polymorphonuclear cells/l sputum). Soluble proteins were isolated from washed sputum plugs (whole sputum) as described (11). In brief, sputum proteins were reduced, and alkylation and intact cells, cellular debris, and insoluble mucins/proteins were removed by centrifugation. The concentration of soluble sputum proteins was measured (Direct Detect, Millipore) and normalized prior to biomolecular characterization*

**Handling of Human Neutrophil-like Cells—**Human promyelocytic leukemia cells (HL-60, ATCC CCL-240) were differentiated (5–6 days, 1.3% (v/v) DMSO, Sigma) to meta/band/segmented neutrophil-like cells. HL-60 cells were cultured (RPMI 1640, Gibco, 10% fetal bovine serum, 2 mM l-glutamine, and 50 units/ml penicillin and 50 μg/ml streptomycin) at 37 °C under 5% CO^2. High differentiation efficiencies (>50%) were obtained as assessed by morphology of Wright-Giemsa stained cells (see below). Cells were washed in PBS before use.

**LC-MS/MS-based N-Glycome Characterization—**N-Glycans were released by 2–5 units of N-glycosidase F (PNGase F, Flavobacterium meningosepticum, Roche Applied Science)/10 μg of proteins (37 °C, 10–12 h) of protein extracts from sputum, human neutrophils, neutrophil-like cells, and P. aeruginosa as described (31) (see supplemental Table 1 for details of sample handling and data acquisition of all LC-MS/MS N-glycome experiments). The resulting N-glycans and N-glycan reference compounds, Dextra Laboratories) were profiled in their hydroxylated state after porous graphitized carbon (PGC) solid phase extraction desalting (31) using PGC-LC (Hyercarb, Thermo Scientific, 5 μm; 180 μm × 10 cm; pore size: 250 Å (LC: Ultimate 3000, Dionex) collision-induced dissociation negative ion MS/MS (HCT three-dimensional ion trap, Bruker Daltonics or LTQ-XX, Agilent).

**LC-MS/MS-based Proteome and Glycoproteome Profiling—**The CF sputum proteome and N-glycoproteome were mapped following a crude fractionation on 4–12% SDS-PAGE (fractions 1–5: 65–100, 50–65, 35–50, 20–35, and 5–20 kDa, respectively). The fractions were digested individually in 20 μl of 50 mM NH_4HCO_3 (aq), pH 7.8, using porcine trypsin (Pro-
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megA, 50 ng/μL, 8 h, 37 °C). The resulting peptide mixtures were used for proteome and glycoproteome mapping. N-Glycopeptides were de-N-glycosylated using 3 units of N-glycosidase F/fraction (12 h, 37 °C) and desalted prior to proteomics or enriched in their intact form using hydrophilic interaction liquid chromatography solid phase extraction prior to N-glycopeptidomics as described (32). All fractions were analyzed individually using C18 LC-Orbitrap Elite-MS/MS (Thermo Scientific) with higher-energy collision-induced dissociation (HCD) and collision-induced dissociation MS/MS fragmentation as described (10) (see supplemental Tables 2–3 for details of sample handling and data acquisition of all LC-MS/MS proteome and glycoproteome experiments).

LC-MS/MS Data Handling—N-Glycans were manually characterized using (i) molecular mass, PGC-LC retention time, and de novo MS/MS sequencing, and (ii) MS/MS spectral and PGC-LC retention time matching to reference compounds (Dextra Laboratories). Proteome HCD-MS/MS data were searched separately against UniProt Homo sapiens and P. aeruginosa PAO1 (Mascot v2.4). N-Glycopeptidome m/z 204.08-filtered HCD-MS/MS spectra were searched against a targeted sputum proteome and N-glycome database (ByonicTM v1.2, Protein Metrics) (10). Protein identifications were filtered to 1% false discovery rate. N-Glycopeptide identifications (≥4 b/y-ions in HCD-MS/MS) were validated using collision-induced dissociation-MS/MS (see supplemental Fig. 1 for N-glycopeptide annotation). Accurate MS-based relative quantitation of proteins, N-glycans, and N-glycopeptides was performed (33).

Western Blotting of Paucimannosidic Proteins—Paucimannosidic proteins derived from sputum were visualized using paucimannose-recognizing Mannitou IgM (12) (undiluted concentration, 8–10 h) and HRP-conjugated anti-mouse IgM (Life Technologies) (1:3000, 1 h) on SDS-PAGE-separated sputum proteins (10 μg) transferred to nitrocellulose membranes (Bio-Rad). HRP chemiluminescent substrates (Millipore) were used in a ChemiDoc MP system (Bio-Rad).

Substrate Specificity of Hex A—The substrate specificity of human Hex A (SF21 baculovirus-derived, R&D Systems, (Bio-Rad). HRP chemiluminescent substrates (Millipore) were used for proteome and glycoproteome mapping. N-Glycopeptides were de-N-glycosylated using 3 units of N-glycosidase F/fraction (12 h, 37 °C) and desalted prior to proteomics or enriched in their intact form using hydrophilic interaction liquid chromatography solid phase extraction prior to N-glycopeptidomics as described (32). All fractions were analyzed individually using C18 LC-Orbitrap Elite-MS/MS (Thermo Scientific) with higher-energy collision-induced dissociation (HCD) and collision-induced dissociation MS/MS fragmentation as described (10) (see supplemental Tables 2–3 for details of sample handling and data acquisition of all LC-MS/MS proteome and glycoproteome experiments).

The sequence homology of Hex A was assessed by monitoring the N-glycan profiles (above) of bovine fetuin, human IgG, and bovine ribonuclease B (Sigma) with and without inoculation of P. aeruginosa PAO1 and PASS1–3 (10^5 bacteria/μg of protein, 12 h, 37 °C).

Temporal Gene Expression of Paucimannosidic Proteins and Enzymes—Maturation stage-specific expression of genes coding for paucimannosidic biosynthetic enzymes and proteins was investigated using a transcriptional profile of terminal granulocytic human neutrophil differentiation following bone marrow and peripheral blood collection from healthy individuals and granulocyte isolation using density gradient centrifugation and immune-magnetic sorting (GEO accession number GSE19556, platform GPL96). The promyelocytic and myelocytic expression levels of genes coding for putative paucimannosidic enzymes, i.e. HEMA (ID 215155_at/201765_s_at), HEBX (201944_at), MAN2A2 (219999_at), MAN2B2 (214703_s_at), and MANBA (203778_at), and proteins, i.e. AZULI (214575_s_at), MPO (203949_at), CD63 (200663_at), and LAMP2 (200821_at/203042_at/203041_s_at) were represented as a -fold change relative to levels in mature resting human neutrophils.

Pathogen-induced Paucimannosidic Protein Secretion—Pathogen-induced secretion of paucimannosidic proteins was monitored by inoculating DMSO-differentiated HL-60 cells (1–3 h, 37 °C, n = 3) and neutrophil-rich pathogen-free whole sputum (8 h, 37 °C, n = 4) with and without P. aeruginosa PAO1 and PASS1. N-Glycan profiling (above) was performed on acetone-precipitated proteins (80% (v/v), 8 h) secreted into the culture medium (1:100 HL-60:bacteria cell ratio). For sputum neutrophils, 10^5 bacteria/μg of whole sputum was used. Bacteria were estimated based on optical density (assuming A_{500} 0.2 ~ 7.8 × 10^7 CFU/ml). Bacteria and HL-60 co-cultures were performed in serum- and antibiotics-free RPMI 1640 media (37 °C, 5% CO_2, gentle agitation). Cell counts and viabilities were monitored using an electronic cell counter (Bio-Rad) and trypan blue exclusion. Phorbol myristate acetate (PMA) treatment (200 nM, 1–3 h, 37 °C) of HL-60 cells served as activation controls. HL-60 morphology was monitored using Wright-Giemsa-stained smears prepared with a Cytospin centrifuge (Shandon).

MBL Binding Assay—Binding of paucimannosidic proteins derived from pathogen-positive sputum and commercially sourced N-glycans (Dextra Laboratories) to agarose-conjugated mannose-binding lectin (MBL) (Thermo Scientific) was assessed in 300 μL of 20 mM CaCl_2, 1.25 mM NaCl, 10 mM Tris-
RESULTS

Paucimannosylation, the Fourth Type of Human N-Glycosylation—Among other N-glycan alterations, we recently suggested that inflamed pathogen-infected sputum of individuals with CF and URTI displayed paucimannose-rich N-glycome signatures (43.4 ± 4.1 and 27.8 ± 4.4%, respectively) relative to sputum from pathogen-free, but neutrophil-positive, lungs of individuals suffering from pneumonia and chronic obstructive pulmonary disease (3.3 ± 1.3%, p = 2.7–9.7 × 10⁻³) (11) (as summarized in Fig. 1A). Mucoid/non-mucoid P. aeruginosa, A. fumigatus, S. pneumoniae, and S. aureus were identified in the pathogen-infected paucimannose-rich sputum (Table 1), illustrating a pathogen species-unspecific link to paucimannosylation.

Herein, we undertake a thorough investigation of this indication of human protein paucimannosylation by performing in-depth spatio-temporal analyses of the structure, function, and biosynthesis of paucimannosidic proteins in neutrophil-rich sputum from pathogen-infected individuals, isolated blood-derived human neutrophils, and neutrophil-like cells (HL-60). The detailed structures and distribution of five chromatographically pure paucimannosidic N-glycans (M1F, M2, M2F, M3, and M3F) were determined in pathogen-infected sputum (Fig. 1B). M2F (Manα1,6Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc) was consistently the most abundant paucimannosidic N-glycan; the corresponding α1,3-isomer of M2F (and M2) was absent. The detailed N-glycan characterization was facilitated by de novo MS/MS sequencing and by spectral and PGC-LC retention time matching to paucimannosidic reference compounds (Fig. 1C, see supplemental Table 1 for supporting N-glycome data). Although M0F per se does not fall under our definition of pauci-
mannosylation, the presence of this paucimannosidic-related structure was confirmed in the CF sputum. Trace levels of the truncated M1, M0, (Fuc\(\alpha\)H9251 1,6)GlcNAc, or single GlcNAc residues may have been present below the detection limit or not released efficiently by \(N\)-glycosidase F. In support of these observations, Western blotting using the paucimannose-reactive Mannitou antibody showed high reactivity to CF and URTI sputum proteins (Fig. 1D).

Possible exogenous bacterial origins of the abundant paucimannosylation in sputum were ruled out by the absence of paucimannosidic \(N\)-glycan signatures of proteomes obtained from isolated and cultured laboratory wound (PAO1) and CF (PASS1–2) P. aeruginosa strains. In addition, no significant \(\alpha\)-sialidase, \(\beta\)-galactosidase, \(\beta\)-hexosaminidase, \(\alpha\)-fucosidase, and \(\alpha\)-mannosidase activities were detected in any of the investigated P. aeruginosa strains using a series of digestion assays with well characterized glycoproteins displaying a spectrum of glycoepitopes and LC-MS/MS \(N\)-glycan profiling, thus confirming that sputum paucimannosylation does not result from exogenous P. aeruginosa exoglycosidase activities.

**Granule-specific Paucimannosylation of Human Neutrophil Proteins**—LC-MS/MS-based proteome mapping of CF sputum revealed the protein characteristics of significant leukocytes, e.g. abundance of MPO, neutrophil elastase (NE), eosinophil peroxidase, lactoferrin, catalase, and aminopeptidase N (see supplemental Table 2 for more). Graphics modified and used with permission from Blausen Medical (Blausen Gallery 2014).

![Pathogen-infected sputum and human neutrophils share biomolecular characteristics. A, similar proteomes from CF sputum (blue) and human neutrophils (red) (*, see Ref. 34). B, near identical \(N\)-glycomes of proteins derived from CF sputum (blue) and blood-derived resting human neutrophils (red) (mean \pm S.D.) (see inset for correlation, \(R^2\); A, NeuAc; H, Hex; N, GlcNAc; F, fucose. C, the granular distribution of predicted \(N\)-glycoproteins identified in CF sputum using neutrophilic granule libraries (34) shows that the putative glycoproteins (when not considering their type of \(N\)-glycosylation) localize to all four main compartments of the human neutrophil, but preferentially to the azurophilic granules (red) (see supplemental Tables 1 and 2 for more).](image-url)

**TABLE 1**

| P. aeruginosa strain | Tissue origin | Patient origin* | Strain origin | Type | Biofilm formation⁵ | Genome known |
|----------------------|--------------|-----------------|--------------|------|-------------------|-------------|
| PAO1                 | Wound        | NA*             | Laboratory   | Non-mucoid | Yes               | Yes         |
| PASS1                | Sputum       | CF5             | Clinical     | Mucoid   | Yes               | Yes⁶        |
| PASS2                | Sputum       | CF1             | Clinical     | Mucoid   | No                | Yes⁶        |
| PASS3                | Sputum       | CF2             | Clinical     | Non-mucoid | Yes               | Yes⁶        |
| PASS4                | Sputum       | CF4             | Clinical     | Mucoid/Non-mucoid | Yes | Yes⁶ |

* See Ref. 11 for de-identified sputum donor information.

† Determined on a flow cell system on LB and minimal media with glucose (data not shown).

‡ NA, not applicable.

§ Recently genome-sequenced and functionally characterized by Prof. Ian Paulson, Macquarie University, Sydney, Australia (unpublished).
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**TABLE 2**

Human paucimannosidic proteins and their relative glycoform distribution identified in pathogen-infected sputum using glycoproteomics

| Protein                        | UniProt   | Asn site | \(\Sigma_{\text{pauc}}\) (%) | Paucimannosidic glycans (%) | M3F | M3 | M2F | M2 | M1F |
|--------------------------------|-----------|----------|-----------------------------|-----------------------------|-----|----|-----|----|-----|
| MPO                            | P05164    | 323      | 63.0                        | 13.5                        | 3.9 | 39.9 | 5.6 | 0.2 |
| \(\beta\)-Glucuronidase         | P08236    | 355      | 0.6                         | 67.3                        | 2.2 |
| LAMP2                          | P13473    | 483      | 69.5                        | 73.0                        | 11.1 | 2.1 |
| AZU                           | P20160    | 356      | 100                         | 100                         | 100 |
| NE                             | P08246    | 272      | 86.1                        | 100                         | 100 |
| LAMAN                          | O00754    | 692      | 100                         | 100                         | 100 |
| Azurocidin                     | P20160    | 171      | 100                         | 100                         | 100 |
| CEACAM6                        | P40199    | 197      | 100                         | 100                         | 100 |
| CD63                           | P08962    | 130      | 100                         | 100                         | 100 |
| CREG1                          | O75629    | 160      | 67.0                        | 100                         | 100 |
| \(\alpha\)-1-Antitrypsin       | P01011    | 186      | 100                         | 100                         | 100 |
| Aspartylglucosaminidase         | P20933    | 38       | 54.1                        | 20.2                        | 34.0 |
| Glutaminyl hydrolase           | Q92820    | 203      | 73.6                        | 20.2                        | 53.3 |
| NGA1*                          | P80188    | 85       | 0.2                         | 0.1                         | 0.1 |
| Phospholipase B-like 1         | Q6P4A8    | 71       | 10.2                        | 10.2                        | 10.2 |
| \(\alpha\)-1-Antitrypsin       |            | 366      | 44.0                        | 44.0                        | 44.0 |
| Ig \(\alpha\)-2 chain C         | P01877    | 205      | 1.6                         | 1.6                         | 1.6 |
| Ig \(\mu\)-chain C              | P01871    | 46       | 7.7                         | 7.7                         | 7.7 |
| UPF0762 protein C6orf58        | Q0P7S2    | 69       | 3.1                         | 3.1                         | 3.1 |

*NGAL, neutrophil gelatinase-associated lipocalin.

human neutrophil proteins \(R^2 = 0.90\); in particular, strong correlation of the paucimannosidic \(\text{N}\)-glycans \(R^2 = 0.96\) was observed (Fig. 2B).

Utilizing sequon-based (NX(T/S), \(X \neq P\)) prediction of \(\text{N}\)-glycosylation and published granule proteome libraries of human neutrophils (34), the putative \(\text{N}\)-glycoproteins of CF sputum were shown to localize to all four main subcellular granular compartments of the human neutrophil, i.e. azurophil, specific, gelatinase/ficolin granules, and secretory vesicles. However, significant proportions of the \(\text{N}\)-glycoproteome \(\sim 40\%\) resided in the azurophilic granule (Fig. 2C).

Unequivocal evidence for human protein paucimannosylation in pathogen-infected sputum and its granule specificity was generated by system-wide mapping of intact glycopeptides using our recently developed glycoproteomics technology (10). Site-specific paucimannosylation was identified on 18 abundant proteins (23 \(\text{N}\)-sites, 35 unique \(\text{N}\)-glycopeptides) of the total of 30 human \(\text{N}\)-glycoproteins identified in CF sputum (36 \(\text{N}\)-sites, 115 unique \(\text{N}\)-glycopeptides), quantitatively covering \(\sim 20\%\) of the CF sputum proteome (Table 2, see also supplemental Table 3 and supplemental Fig. 1 for supporting glycopeptide data). By overlaying these data onto granule proteome libraries of human neutrophils (34), paucimannosylation was found to be highly enriched in the azurophilic granules \(p = 2.3 \times 10^{-5}\) relative to the other three main compartments of the neutrophil (Fig. 3, A and E). High mannose and complex type \(\text{N}\)-glycoproteins displaying \(\beta\)-galactosylation, Lewis type fucosylation, and \(\alpha\)-sialylation localized predominantly to the other granules (Fig. 3, B–E). These observations were supported by partial co-localization of the azurophilic marker MPO and paucimannosidic epitopes in DMSO-differentiated human neutrophil-like cells using immunocytochemistry (data not shown).

Spatio-temporal Paucimannosidic Generation by Human \(\beta\)-Hex A—The biosynthetic mechanisms of human paucimannosylation were investigated. We have previously shown that \(\text{N}\)-glycan processing and the solvent accessibility of \(\text{N}\)-glycosylation sites on maturely folded proteins are closely correlated (37). The identified paucimannosidic \(\text{N}\)-glycosylation sites on sputum proteins were found to be significantly more accessible than the spatially hidden high mannose sites \(p = 8.0 \times 10^{-4}\) showing solvent accessibilities similar to the highly processed complex sites (Fig. 4A), indicating that paucimannosylation results from significant exoglycosidase processing of the solvent-exposed \(\text{N}\)-glycan structures. No specific sequence recognition motifs for the paucimannosidic \(\text{N}\)-glycosylation sites were evident as assessed by a frequency plot.

In paucimannose-rich invertebrates, hexosaminidases are required for the removal of the \(\beta\_1,2\)-GlCNAc residue on the \(\text{3'}\)-mannose arm to form paucimannosidic structures (28). The \(\alpha\) and \(\beta\) subunits of the heterodimeric human Hex A showed high sequence similarities to hexosaminidases of such organisms, in particular to \(\text{C. elegans}\) Hex A \(53.8\%\) and \(52.9\%\) and \(\text{Arabidopsis thaliana}\) Hex1–3 \(49.1\%\) and \(47.9\%\) (Fig. 4B). In vitro incubation of human Hex A with \(\beta\_1,2\)-GlCNAc-terminating \(\text{N}\)-glycan substrates, at physiologically realistic conditions, \(\text{i.e.}\) enzyme/substrate ratio, temperature, and organelle-like pH, generated \(\text{M3F, albeit at low enzymatic rates (Fig. 4C). Furthermore, \(\beta\_1,2\)-GlCNAc-terminating afucosylated complex and hybrid type \(\text{N}\)-glycans and \(\beta\_1,4\)-GalNAC-terminating GM2 glycolipids were found to be acceptable Hex A substrates (Fig. 4D). Hex A showed no activity on \(\beta\_1,2\)-GlCNAc-terminating \(\text{N}\)-glycan substrates when proximal antennas carried \(\beta\_1,4\)-galactosylation or \(\beta\_1,4\)-bisecting GlCNACylation (Fig. 4E).}

Partial co-localization of human Hex A and paucimannosidic glycopeptides as evaluated by immunocytochemistry of DMSO-differentiated human neutrophil-like cells supported the involvement of Hex A in paucimannose production (Fig. 4F). Azurophilic granule residence of human Hex A was indicated by moderate/strong co-localization of Hex A with the azurophilic marker MPO (Fig. 4G). This was supported by proteomics-based identifications of \(\alpha\) and \(\beta\) subunits of Hex A in isolated azurophilic granules of human neutrophils (34, 38).
High expression of the genes coding for the putative paucimannosidic enzymes, e.g. HEXA and HEXB (coding for \( \beta \)-hexosaminidase subunit \( \alpha \) and \( \beta \), respectively, which together hydrolyze \( \beta \)-GlcNAc- and \( \alpha \)-GalNAc-terminating glycoconjugates), and for paucimannosidic proteins, e.g. MPO and AZU1 (coding for MPO and azurocidin, respectively) in promyelocytes, relative to levels in myelocytes and mature (resting) neutrophils, indicated assembly of the synthetic machinery for paucimannosylation (but not necessarily the complete biosynthetic generation of paucimannosidic proteins), early in the bone marrow maturation (Fig. 4H). M3(F) truncation to M2(F), M1(F), and M0(F) may be facilitated by human \( \alpha \)- and \( \beta \)-mannosidases previously identified in the azurophilic granules of human neutrophils (34, 38); promyelocytic stage-specific expression of the corresponding mannosi-dase genes, i.e. MAN2A2/MAN2B2 (coding for \( \alpha \)-mannosidases, which hydrolyze terminal \( \alpha \)1,3/6-linked mannosides) and MANBA (coding for \( \beta \)-mannosidase, which hydrolyzes terminal \( \beta \)-linked mannosides), was indeed observed. Taken together, we propose a new granule- and maturation-specific assembly of the biosynthetic machinery for human protein paucimannosylation in the azurophilic granules during early myeloid maturation of neutrophil precursors in the bone marrow (Fig. 4I).

Pathogen-induced Secretion of Paucimannosidic Proteins—Pathogen-induced secretion of paucimannosidic proteins was observed in vitro from DMSO-differentiated neutrophil-like HL-60 cells (Fig. 5A, top). \( P. \) aeruginosa PAO1 and PASS1 induced a time-dependent (1 h versus 3 h, \( p = 1.8 \times 10^{-3} \)) release of paucimannosidic proteins, preferentially M2F glycoforms (Fig. 5A, bottom), into the culture medium. Upon \( P. \) aeruginosa inoculation, the total levels of paucimannosylation were consistently above the unchallenged secretion levels (\( p = 5.4 \times 10^{-3} \) to \( 5.0 \times 10^{-5} \)); less elevation of the paucimannosylation levels was observed when the neutrophil-like HL-60 cells were activated with PMA (\( p = 0.05 \), 1 h versus 3 h). The unchanged morphology and cell counts of PAO1-challenged (1 h) neutrophils and PMA-activated neutrophils relative to the resting level suggested active secretion of paucimanno-
sidic proteins from viable cells by degranulation mechanisms. However, reduced cell numbers and viability after prolonged bacteria inoculation (2–3 h), in particular with the virulent CF-derived PASS1 strain, indicated that paucimannosidic proteins may, in part, be released into the culture media upon cell death under these conditions. Interestingly, only PASS1 inoculation induced significant secretion (but not M2F-specific secretion) of paucimannosidic proteins into neutrophil-rich pathogen-free sputum (31.9% ± 8.4%) relative to the unchallenged counterpart (9.4% ± 2.7%, p = 2.2 × 10^{-7}, n = 4) (Fig. 5B).
Binding of Paucimannosidic Proteins to Mannose Receptors—

Potential involvement of protein paucimannosylation in complement activation was assessed by evaluating the binding capacity of paucimannosidic glycopeptides to MBL. All paucimannosidic glycoforms carried by the CF sputum proteins showed affinities to MBL (Fig. 5C). Isolated paucimannosidic N-glycans from commercial sources showed similar binding behavior. However, the significant presence of paucimannosidic glycoforms in the MBL unbound fractions indicated low binding affinities or MBL saturation under the assayed conditions. High mannose-containing bovine ribonuclease B did not bind to MBL, whereas free high mannose N-glycans showed significant affinities (data not shown).

DISCUSSION

Augmenting established glycobiology (15–21), we here, for the first time, demonstrate that humans also produce bioactive paucimannosidic proteins similar to lower organisms such as nematodes, insects, and plants (15, 16, 26, 28) (see Fig. 6 for overview of our findings). However, in contrast to lower organ-
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**FIGURE 6. Structure, biosynthesis, and function of paucimannosylation as a fourth type of protein N-glycosylation in human neutrophils.** Shown is a schematic overview illustrating in a simplified form the main findings of this work in terms of structure (N-glycan species and protein carriers), temporal, and spatial aspects of the biosynthetic route, as well as the functions of protein paucimannosylation in the human neutrophil. As such, protein paucimannosylation constitutes a new and fourth type of human N-glycosylation in addition to the established high mannose, hybrid, and complex type N-glycosylation. This implies that human protein paucimannosylation is a fourth type of protein (in addition to high mannose, hybrid, and complex type) in neutrophil-rich environments central to inflammation and infection.

The discovery of this alternate type of human N-glycosylation, as exemplified in pathogen-infected sputum, was enabled by recent analytical developments in system-wide characterization of protein glycosylation (10, 31). Protein paucimannosylation was found to be abundant in sputum from inflamed pathogen-infected lungs irrespective of lung disease/condition, infecting microorganism, gender, age, and antibiotic treatment. Pathogen-free sputum, although derived from neutrophil-rich lungs (39), displayed negligible amounts of protein paucimannosylation. This implies that human protein paucimannosylation is neither genotype-specific, microbe-specific, nor disease-specific, but rather a general molecular feature common to inflamed micro-environments of hosts undergoing pathogenic attack.

Five human paucimannosidic N-glycans were found to be carried by 18 abundant human sputum proteins that localized specifically to the azurophilic granules of the multi-compartmentalized human neutrophil (40). Neutrophilia is well established in CF and other respiratory conditions including URTI, featuring high proportion (>95%), counts (>10^7 cells/g of sputum), and viability (>70%) of neutrophils in sputum (41). Strong support of paucimannosidic subcellular-specific localization in azurophilic granules and the proposed association between sputum protein paucimannosylation and neutrophil activation by pathogens comes from the observation that purified neutrophil proteins, including human MPO (42, 43), proteinase 3 (PR3) (44), azurocidin (45), Hex B (46), and bovine α-mannosidase (47), which localize to azurophilic granules (34), were previously shown to carry monosaccharide compositions corresponding to paucimannosidic N-glycans. Our glycoproteomics data indicate that sputum glycoproteins localizing to the specific and gelatinase granules and secretory vesicles in human neutrophils carried preferentially complex and high mannose N-glycans, suggesting a compartment-specific production and storage of paucimannosidic proteins in azurophilic granules. Highly similar N-glycosylation profiles of pathogen-infected sputum and neutrophil proteins, including the paucimannosidic profiles, further supported the neutrophil origin of paucimannosylation and are congruent with studies indicating mammalian paucimannosylation in cancer and systemic lupus erythematosus (5, 7, 8, 12), which are neutrophil-rich pathologies. The neutrophil N-glycosylation profile in our study resembles a previously reported human neutrophil N-glycan profile (48) in which the low mass paucimannosidic structures however were not reported. The biosynthetically intriguing mono-antennary sialo-N-glycans (NeuAcGal1Man6GlcNAc2Fuc0−1) were abundant in the neutrophil and CF sputum N-glycomes, but did not appear to be directly related to the azurophilic granule-specific paucimannosylation based on their attachment to proteins localizing to other neutrophil granules (supplemental Fig. 1).

The fact that paucimannosidic N-glycans are carried by highly solvent-accessible sites on sputum proteins suggests that they are derived from extensive exoglycosidase processing (37). The high solvent accessibilities also explain the prevalence (>85%) of the accessibility-dependent α1,6-(core) fucosylation on the paucimannosidic N-glycans. High core fucosylation, in turn, implies that the paucimannosidic biosynthetic route involves N-glycan intermediates displaying terminal β1,2-GlcNAcylation, a substrate requirement for fusocyltransferase 8 (49). This implies again that paucimannose generation follows the initial synthesis of cis-Golgi-localized fusocyslated hybrid/complex glycan intermediates.

Hexosaminidases are highly expressed in paucimannose-rich organisms including *C. elegans*, *D. melanogaster*, and plants (21). We observed high sequence homology of the α and β subunits of the heterodimeric human Hex A to these hexosaminidases in line with a previous study (20). In addition, the specific identification of human Hex A α/β subunits in azurophilic granules of neutrophils (34, 38) and our immunocyto-
chemistry data showing partial co-localization of both human Hex A and paucimannosidic glycoepitopes with an azurophilic granule marker (MPO) in differentiated neutrophil-like HL-60 cells together support the Hex A-driven compartment-specific paucimannosylation pathway proposed herein. The capacity of Hex A to generate paucimannosidic N-glycans in vitro from biosynthetic intermediates at realistic physiological conditions, albeit at low enzymatic rates, also confirms this relationship. The similarities of primary (66.5% sequence similarity) (data not shown) and higher structural levels of α and β subunits of Hex A (50, 51) suggest that the homodimeric Hex B (ββ) and Hex S (αα) isoenzymes may also be able to catalyze paucimannosylation. The relative broad substrate specificity of hexosaminidases to both α- and β-galactosidases and α-sialidases and β-galactosidases in azurophilic granules from the azurophilic compartment of the human neutrophil (58, 59), displayed complex type N-glycosylation. This agrees well with the exclusive presence of the α,1,6-mannose isomer of M2F and M2 isomers (see supplemental Table 1). This agrees well with the exclusive presence of the α,1,6-mannose isomer of M2F and M2 previously reported on neutrophil-derived human proteins (45) and the preferential hydrolysis of α,1,3-linked mannoses by human α-mannosidase (61). Human β-mannosidase may be responsible for yielding aman- nosylated di- (M0) or tri- (M0F) saccharides. The detection of α- and β-mannosidases in isolated azurophilic granules (34, 38) and the promyelocyte-specific expression of genes coding for the corresponding mannosidases support their association with protein paucimannosylation.

Assembly of the paucimannose generating azurophilic granules and its molecular components early during myeloid maturation in the bone marrow was supported by the temporal gene expression of paucimannosidic proteins and putative paucimannose biosynthetic enzymes in promyelocytes in excellent agreement with previous studies (55, 56). Gene set enrichment analysis (57) revealed a high gene expression of paucimannosidic proteins in isolated blood leukocytes from S. pneumoniae-infected (but not S. aureus, Escherichia coli, and influenza A virus-infected) individuals (9-fold enrichment, p = 3.4 × 10−3) relative to healthy individuals, suggesting that the assembly of the paucimannose biosynthetic machinery in neutrophil precursors can be shifted from the bone marrow to the blood circulation via infection-dependent “left shifts.”

The “targeted-by-timing biosynthesis” hypothesis mechanistically explaining the formation of the granule-specific proteomes in neutrophils (34, 40, 58) is congruent with our observation of compartment-specific N-glycosylation; the majority of all glycoproteins trafficking through the N-glycosylation machinery at the promyelocytic stage of the neutrophil development are directed to the azurophilic granules by vesicles budding from the cis-Golgi without reaching the late N-glycan maturation stage, e.g. β-galactosylation and α-sialylation in the trans-Golgi network (59). As expected, proteins localizing to the specific (and other) granules, which are synthesized exclusively in the myelocyte and more mature stages of the neutrophil development by vesicles budding off from the trans-Golgi network (58, 59), displayed complex type N-glycosylation. This subcellular-specific N-glycosylation is further supported by the absence of human α-sialidases and β-galactosidases in azurophilic granules (34, 38), which suggest that Hex A does not function in concert with other outer-arm exoglycosidases and may explain why the sterically protected GlcNAc-terminating N-glycans are unacceptable substrates for human Hex A. This fascinating feature of compartment-specific N-glycosylation is not unique to neutrophils (60).

The further trimming of the paucimannosidic glycoforms by human α-mannosidases appears to be linkage-specific as shown by the specific generation of the α,1,6-linked mannose-terminating M2F and M2 isomers (see supplemental Table 1). This agrees well with the exclusive presence of the α,1,6-mannose isomer of M2F and M2 previously reported on neutrophil-derived human proteins (45) and the preferential hydrolysis of α,1,3-linked mannoses by human α-mannosidase (61). Human β-mannosidase may be responsible for yielding aman nosylated di- (M0) or tri- (M0F) saccharides. The detection of α- and β-mannosidases in isolated azurophilic granules (34, 38) and the promyelocyte-specific expression of genes coding for the corresponding mannosidases support their association with protein paucimannosylation.

3 A. C. Dahmen, M. T. Fergen, C. Laurini, B. Schmitz, I. Loke, M. Thaysen-Andersen, and S. Diestel, submitted for publication.
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N-glycome), supporting a possible oncofetal antigen potential of paucimannosylation (12) and the possible molecular and functional similarity of neutrophils and epithelial cells (58). However, the lack of azurophilic granules in epithelial cells implies that production, storage, and secretion of paucimannosidic proteins may be facilitated by other mechanisms in these systems.

Containing an ensemble of bioactive molecules, including antimicrobial peptides and proteases, the azurophilic granule is the microbicidal compartment of the neutrophil (62, 63). Thus, the azurophil-specific localization of protein paucimannosylation in neutrophils is potentially of high biological significance. It has been established that azurophilic granules are mobilized as the last compartment upon phagocytosis (40, 62, 63), emptying their soluble content into the phagolysosome and the extracellular environment to combat invading pathogens (40). The virulence-specific release of paucimannosidic proteins into sputum upon P. aeruginosa stimulation indicates an infection-dependent mobilization of azurophilic granules, aspects we are currently investigating by bacterial genome sequencing and proteomics. The importance of granule mobilization for innate immunity is well illustrated in the Chediak-Higashi syndrome where immobile azurophilic granules reduce the host response to pathogens (64). The induced secretion of paucimannosidic proteins from neutrophils presented here indicates that, as reported (40, 62), granules fuse not only with the phagolysosome, but also with the plasma membrane upon activation.

The strong neutrophilic association with the paucimannosidic proteins observed in sputum from pathogen-infected inflamed lungs prompted us to investigate possible functional aspects of human paucimannosylation, i.e. lectin-based recognition by the immune system via mannose-receptor interactions. Mannose-terminating glycoconjugates are infrequent in the extracellular environment of healthy human cells/tissues (60), but such determinants from internal membranes or granules may be exposed under specific cellular conditions, e.g. immature ER-resident glycopeptides were shown to be exposed in apoptotic cells serving as “eat me” signals for macrophage-based clearance (65). Exposure of α- and β-mannose determinants on solvent-accessible glycosylation sites such as those presented by paucimannosidic proteins may be a unique feature to enable cellular and cellular communication of activated neutrophils via mannose receptors in the micro-environment by mechanisms of active secretion (degranulation) of paucimannosidic proteins or by release upon cell death (39). The abundant paucimannosidic determinants found in infected sputum may also arise from the release of granular contents into neutrophil extracellular traps (NETs) via the activation and NETosis of polymophonuclear cells (66). We have demonstrated binding of paucimannosidic proteins and N-glycans to MBL; however, the exact roles of paucimannosylation in the downstream complement activation clearly need further investigation. In addition, paucimannosidic binding to other lectins including the macrophage mannose receptors (CD206 and CD280) and dendritic C type lectins (e.g. CD209, CD299, and CD303) may provide avenues for the neutrophils to communicate with other immune cells (67). Opportunistic pathogens may also recognize exposed mannosidic determinants as an avenue for host adherence, e.g. E. coli fimbrial FimH adhesin shows high affinity for high mannose type as well as paucimannosidic N-glycans (68, 69).

In addition, bacteriostatic and bactericidal effects of the paucimannose-rich neutrophilic proteins NE, azurocidin, and cathepsin G have very recently been reported (70). We are currently investigating the functional roles of the carbohydrate moieties on these human paucimannosidic proteins in the context of bacterial killing and growth inhibition.

Finally, we speculate that paucimannosylation may carry out modulatory roles in the generation and/or recognition of anti-neutrophil cytoplasmic autoantibodies by masking or presenting immunogenic epitopes on the anti-neutrophil cytoplasmic autoantibody-typic and paucimannose-rich LAMP2, PR3, MPO, and NE (71). As such, it becomes clear that paucimannosylation may be linked to multiple diverse functional roles in the micro-environments where paucimannosidic proteins appear to be enriched following neutrophil activation, i.e. in phagolysosomes (39, 62), microvesicles/ectosomes (72) and neutrophil extracellular traps (73).

In conclusion, we document that human neutrophils produce, store, and selectively secrete bioactive paucimannosidic proteins into sputum of lungs undergoing pathogen-based inflammation and infection. We show that the azurophilic granules of neutrophils are the biosynthetic “venues” and that human hexosaminidases are the enzymatic “facilitators” of this fourth type of human protein N-glycosylation. In line with established neutrophil biology, we propose that paucimannosidic proteins are targeted to the azurophilic granule “by timing” rather than by selective sorting in the early bone marrow maturation of developing myeloid cells. The rather narrow temporal and spatial nature of paucimannosidic proteins in micro-environments surrounding inflammation, and in response to pathogen infection, suggests specialized biomolecular immune functions and may explain how protein paucimannosylation to date has remained under the radar in human glycobiology.

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Note Added in Proof—Fig. 4F did not indicate that the images of the neutrophil-like HL-60 cells were obtained from separate fields in the version of this article that was published on February 2, 2015 as a Paper in Press. This error has been corrected.

REFERENCES
1. Freeze, H. H. (2013) Understanding human glycosylation disorders: biochemistry leads the charge. J. Biol. Chem. 288, 6936–6945
2. Moremen, K. W., Tiemeyer, M., and Nairn, A. V. (2012) Vertebrate protein glycosylation: diversity, synthesis and function. Nat. Rev. Mol. Cell
Human Paucimannosylation Structure, Function, and Biosynthesis

Biol. 13, 448 – 462
3. Aebi, M. (2013) N-linked protein glycosylation in the ER. Biochim. Biophys. Acta 1833, 2430 – 2437
4. Everest-Dass, A. V., Jin, D., Thaysen-Andersen, M., Nevalainen, H., Kola-rich, D., and Packer, N. H. (2012) Comparative structural analysis of the glycosylation of salivary and buccal cell proteins: innate protection against infection by Candida albicans. Glycobiology 22, 1465–1479
5. Balog, C. I., Stavenhagen, K., Fung, W. L., Koeleman, C. A., McDonnell, L. A., Verhoeven, A., Mesker, W. E., Tollenaar, R. A., Deelder, A. M., and Wührer, M. (2012) N-Glycosylation of colorectal cancer tissues: a liquid chromatography and mass spectrometry-based investigation. Mol. Cell. Proteomics 11, 571 – 585
6. Sethi, M. K., Thaysen-Andersen, M., Smith, J. T., Baker, M. S., Packer, N. H., Hancock, W. S., and Fanayan, S. (2014) Comparative N-glycan profiling of colorectal cancer cell lines reveals unique bisecting GlcNaC and α-2,3-linked sialic acid determinants are associated with membrane proteins of the more metastatic/aggressive cell lines. J. Proteome Res. 13, 277– 288
7. Joosten, C. E., Cohen, L. S., Ritter, G., Batt, C. A., and Shuler, M. L. (2004) Prog. Carbohydr. Res. 39, 559–576
8. Hashlam, S. M., Gems, D., Morris, H. R., and Dell, A. (2002) The glycomes of Caenorhabditis elegans and other model organisms. Biochem. Soc. Symp. 67 – 134
9. Parker, B. L., Thaysen-Andersen, M., Solis, N., Scott, N. E., Larsen, M. R., Kola-rich, D., and Boulianne, G. L. (2006) Null mutations in α2,6-sialic acid saminidase probably involved in the processing of protein N-glycans. J. Biol. Chem. 270, 17344–17349
10. Peter, B. L., Thaysen-Andersen, M., Solis, N., Scott, N. E., Larsen, M. R., Graham, M. E., Packer, N. H., and Cordwell, S. J. (2013) Site-specific glycan- peptide analysis for determination of N-glycoprotein heterogeneity. J. Proteome Res. 12, 5791–5800
11. Venkatakrishnan, V., Thaysen-Andersen, M., Chen, S. C., Nevalainen, H., and Packer, N. H. (2015) Cystic fibrosis and bacterial colonization define the sputum N-glycosylation phenotype. Glycobiology 25, 88–100
12. Zipper, B., Bello-DeOcampo, D., Diestel, S., Tai, M. H., and Schmitz, B. (2012) Mannitou monoclonal antibody uniquely recognizes paucimannose, a marker for human cancer, stemness, and inflammation. J. Carbohydr. Chem. 31, 504–518
13. van Remoortere, A., Verhoeven, A., Mesker, W. E., Tollenaar, R. A., Deelder, A. M., and Boulianne, G. L. (2011) Life is sweet! A novel role for Caenorhabditis elegans N-glycosylation. J. Biol. Chem. 286, 4867–4875
14. Gutternigg, M., Kretschmer-Lubich, D., Paschinger, K., Rendić, D., Hader, J., Geier, P., Ranftl, R., Jantsch, V., Lochnit, G., and Wilson, I. B. (2007) Biosynthesis of truncated N-linked oligosaccharides results from non-orthologous hexosaminidase-mediated mechanisms in nematodes, plants, and insects. J. Biol. Chem. 282, 27825–27840
15. Shacter, H., and Boulianne, G. (2011) Life is sweet! A novel role for N-glycans in Drosophila lifespan. Fly 5, 18–24
16. Léonard, R., Rendić, D., Rabouille, C., Wilson, I. B., Prêt, J., and Altman, F. (2006) The Drosophila fused lobes gene encodes an N-acetylglucosamine involved in N-glycan processing. J. Biol. Chem. 281, 4867–4875
17. Schiller, B., Hykollari, A., Yan, S., Paschinger, K., and Wilson, I. B. (2012) Complicated N-linked glycans in small organisms. Biol. Chem. 393, 661 – 673
18. Joosten, C. E., Cohen, L. S., Ritter, G., Batt, C. A., and Shuler, M. L. (2004) Synthesis of paucimannose N-glycans by Caenorhabditis elegans N-acetylglucosaminidase II and a specific membrane-bound β-N-acetylglucosaminidase. Biochim. J. 372, 53–64
19. Shi, H., Tan, J., and Schacter, H. (2006) N-Glycans are involved in the response of Caenorhabditis elegans to bacterial pathogens. Methods Enzymol. 417, 359–389
20. Abbas, A. R., Baldwin, D., Ma, Y., Ouyang, W., Gurney, A., Martin, F.,
37. Thaysen-Andersen, M., and Packer, N. H. (2012) Site-specific glycoproteomics confirms that protein structure dictates formation of N-glycan type, core fucosylation and branching. Glycobiology 22, 1440–1452
38. Lominadze, G., Powell, D. W., Luerman, G. C., Link, A. J., Ward, R. A., and McLeish, K. R. (2005) Proteomic analysis of human neutrophil granules. Mol. Cell. Proteomics 4, 1503–1521
39. Nauseef, W. M., and Borregaard, N. (2014) Neutrophils at work. J. Biol. Chem. 289, 3040–345
40. Borregaard, N., and Cowland, J. B. (1997) Granules of the human neutrophil polymorphonuclear leukocyte. Blood 89, 3503–3521
41. Jayaram, L., Labiris, N. R., Efthimiadis, A., Valchos-Mayer, H., Hargreave, F. E., and Freitag, A. P. (2007) The efficiency of sputum cell counts in cystic fibrosis. Can Respir. J. 14, 99–103
42. Van Antwerpen, P., Slomiany, M. C., Boudjeltia, K. Z., Delporte, C., Faid, V., Calay, D., Rousseau, A., Moguilevsky, N., Raes, M., Vanhamme, L., Furtmüller, P. G., Obinger, C., Vanhaeverbeek, M., Nève, J., and Michalski, J. C. (2010) Glycosylation pattern of mature dimeric leukocyte and recombining monomeric myeloperoxidase: glycosylation is required for optimal enzymatic activity. J. Biol. Chem. 285, 16351–16359
43. Van, T., Houn, G., and Hoff, P. (2010) The glycoseylation of myeloperoxidase. Biochim. Biophys. Acta 1804, 2046–2053
44. Babu, P., North, S. J., Jang-Lee, J., Chalabi, S., Mackerness, K., Stowell, S. R., Paschinger, K., Staudacher, E., Stemmer, U., Fabini, G., and Wilson, I. B. (2010) Site-specific glycosylation analysis of the bovine lysosomal α-mannosidase. Glycobiology 20, 496–503
45. Olczak, M., and Watorek, W. (2002) Structural analysis of N-glycans from human neutrophil azurocin. Biochem. Biophys. Res. Commun. 293, 213–219
46. Schuette, C. G., Weisgerber, J., and Sandhoff, K. (2001) Complete analysis of the glycoseylation and disulphide bond pattern of human β-hexosaminidase B by MALDI-MS. Glycobiology 11, 549–556
47. Faid, V., Eijgen, G., Tollersted, U. K., Michalski, J. C., and Morelle, W. (2006) Site-specific glycosylation analysis of the bovine lysosomal α-mannosidase. Glycobiology 16, 440–461
48. Babu, P., North, S. J., Jang-Lee, J., Chalabi, S., Mackerness, K., Stowell, S. R., Cummings, R. D., Rankin, S., Dell, A., and Haslam, S. M. (2009) Structural characterisation of neutrophil glycans by ultra sensitive mass spectrometric glycoscimics methodology. Glycoconjug. J , 297, 957–986
49. Paschinger, K., Staudacher, E., Stemmer, U., Fabini, G., and Wilson, I. B. (2005) Fucosyltransferase substrate specificity and the order of fucosylation in invertebrates. Glycobiology 15, 463–474
50. Lemieux, M. J., Mark, B. L., Cherney, M. M., Withers, S. G., Mahurin, D. J., and James, M. N. (2006) Crystallographic structure of human β-hexosaminidase A: interpretation of Tay-Sachs mutations and loss of Gcα2 ganglioside hydrolisys. J. Mol. Biol. 359, 913–929
51. Mark, B. L., Mahurin, D. J., Cherney, M. M., Zhao, D., Knapp, S., and James, M. N. (2003) Crystal structure of human β-hexosaminidase B: understanding the molecular basis of Sandhoff and Tay-Sachs disease. J. Mol. Biol. 327, 1093–1109
52. Hepbildikler, S. T., Sandhoff, R., Kolmer, Z., Proia, R. L., and Sandhoff, K. (2002) Physiological substrates for human lysosomal β-hexosaminidase S. J. Biol. Chem. 277, 2562–2572
53. Pillay, J., den Braber, I., Vrieseop, N., Kwant, L. M., de Boer, R. J., Borghini, J. A., Telseeaa, K., and Koenderman, L. (2010) In vivo labeling with 3H₂O reveals a human neutrophil lifespan of 5.4 days. Blood 116, 625–627
54. Meier, E. M., Schwarzmann, G., Fürst, W., and Sandhoff, K. (1991) The human Gcα2 activator protein. A substrate specific cofactor of β-hexosaminidase A. J. Biol. Chem. 266, 1879–1887
55. Martinelli, S., Urosevic, M., Daryadel, A., Oberholzer, P. A., Baumann, C., Fey, M. F., Dummer, R., Simon, H. U., and Yousefi, S. (2004) Induction of genes mediating interforeron-dependent extracellular trap formation dur-