G6PC3 mutations are associated with a major defect of glycosylation: a novel mechanism for neutrophil dysfunction

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Glucose-6-phosphatase, an enzyme localized in the endoplasmic reticulum (ER), catalyzes the hydrolysis of glucose-6-phosphate (G6P) to glucose and inorganic phosphate. In humans, there are three differentially expressed glucose-6-phosphatase catabolic genes (G6PC1–3). Recently, it has been shown that mutations in the G6PC3 gene result in a syndrome associating congenital neutropenia and various organ malformations. The enzymatic function of G6PC3 is dependent on G6P transport into the ER, mediated by G6P translocase (G6PT). Mutations in the gene encoding G6PT result in glycogen storage disease type-1B (GSD-1B). Interestingly, GSD-1B patients exhibit a similar neutrophil dysfunction to that observed in G6PC3-deficient patients. To better understand the causes of neutrophil dysfunction in both diseases, we have studied the neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of patients with G6PC3 and G6PT syndromes. Unexpectedly, sodium dodecyl sulfate–polyacrylamide gel electrophoresis experiments indicated hypo-glycosylation of gp91phox, the electron-transporting component of the NADPH oxidase, in all of these patients. Rigorous mass spectrometric glycomic profiling showed that most of the complex-type antennae which characterize the neutrophil N-glycome of healthy individuals were severely truncated in the patients’ neutrophils. A comparable truncation of the core 2 antenna of the O-glycans was also observed. This aberrant neutrophil glycosylation is predicted to have profound effects on the neutrophil function and merit designation of both syndromes as a new class of congenital disorders of glycosylation.

Keywords: G6PC3 / glycogen storage disease type-1b / neutrophil / glycosylation / respiratory burst

Introduction

Glucose-6-phosphatase (EC 3.1.3.9) catalyzes the hydrolysis of glucose-6-phosphate (G6p) to glucose and inorganic phosphate. It is located in the endoplasmic reticulum (ER) membrane with its active site facing the ER lumen (Pan et al. 1998). In humans, there are three differentially expressed glucose-6-phosphatase genes. Glucose-6-phosphatase catalytic-1 (G6PC1) is expressed in the major gluconeogenic organs (liver, kidney and small intestine; Mithieux et al. 1996; Rajas et al. 1999) and plays a key role in overall glucose homeostasis (Mithieux 1997; Mithieux et al. 2004; Pocai et al. 2005). Mutations in G6PC1 cause glycogen storage disease type-1a (GSD-1a) that is characterized by growth retardation, hypoglycemia, hepatomegaly, kidney enlargement, hyperlipidemia, hyperuricemia and lactic acidemia (Lei et al. 2005). Mutations in G6PC3 cause glycogen storage disease type-1b (GSD-1b) that is characterized by growth retardation, hypoglycemia, hepatomegaly, kidney enlargement, hyperlipidemia, hyperuricemia and lactic acidemia (Lei et al. 1993; Chou et al. 2010). G6PC2 is exclusively expressed in pancreatic islet cells (Arden et al. 1999; Martin et al. 2001) and may be involved in glucose-dependent insulin secretion by controlling free glucose levels (Petrolonis et al. 2004). The functions of the third family member, G6PC3, are poorly understood, although it is known to be ubiquitously expressed (Martin et al. 2002; Guionie et al. 2003).

Recently, studies of diseases that involve neutrophil dysfunction have begun to cast some light on the role of G6PC3. Significantly, it has been found that G6PC3 is the defective
gene in a subset of patients with severe congenital neutropenia (Boztug et al. 2009). The neutrophils of these patients display enhanced ER stress and increased rates of apoptosis (Aróstegui et al. 2009; Boztug et al. 2009). Moreover, patients with this syndrome have short stature and cardiac defects. They do not, however, have the metabolic disorders associated with GSD-1a, presumably because their G6PC1 is normal implying that their major gluconeogenic organs have a fully functioning glucose-6-phosphatase. A recent study identified that homozygous G6PC3 G260R mutation abolishes enzymatic function causing neutropenia but without a failure of neutrophil production (McDermott et al. 2010).

G6P, the substrate for glucose-6-phosphatase, is transported into the ER by G6P translocase (G6PT; Pan et al. 1999). G6PT and G6PC appear to work in concert to maintain glucose homeostasis in gluconeogenic organs (Lei et al. 1996; Chou et al. 2002; Boztug and Klein 2009). Mutations in the gene encoding G6PT (Annabi et al. 1998; Chou et al. 2002) cause GSD-1b (Narisawa et al. 1986; Melis et al. 2005), which shares the GSD-1a symptoms. However, in contrast to GSD-1a, GSD-1b additionally exhibits congenital neutropenia and neutrophil dysfunction resulting in impaired neutrophil respiratory burst, defective bacterial killing and consequent susceptibility to infection. Moreover, mice lacking glucose-6-phosphatase-β (equivalent to G6PC3) display a similar neutrophil dysfunction and phenotypic features (Cheung et al. 2007). Responsible for the latter neutrophil respiratory burst is the multimeric enzyme complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which contains both the cytosolic and transmembrane components (Lambeth 2004).

In order to better understand the causes of neutrophil dysfunction in the G6PC3 and G6PT syndromes, we have undertaken an investigation of the NADPH oxidase in the neutrophils of five patients with G6PC3 mutations and two with defects in the gene for G6PT. Unexpectedly, we found that gp91phox, the electron-transporting subunit of the neutrophil NADPH oxidase, ran aberrantly on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels consistent with hypo-glycosylation. In order to better understand this hypo-glycosylation, we employed high-sensitivity mass spectrometric (MS) methodologies to profile the N- and O-glycomes of the patients’ neutrophils. Remarkably, these analyses showed that the complex-type antennae of most of the N- and O-glycans were severely truncated. This aberrant glycosylation was found in all patients and is likely to be the major cause of neutrophil dysfunction.

Results

G6PC3 mutation analysis

Five patients from four unrelated families were studied. There was a common phenotype of short stature, neutropenia and susceptibility to bacterial infection (Table I). In addition to two previously reported mutations in G6PC3, two novel mutations were identified, resulting in amino acid substitution (Pro44Ser; patient C; Figure 1A) or deletion (Thr64-Ile70, siblings A and B; Figure 1A and B). All mutations were predicted to involve transmembrane helices of G6PC3 (Figure 1C).

The neutrophil respiratory burst and cell-free assay

In keeping with observations in glucose-6-phosphatase β-deficient mice (Cheung et al. 2007), superoxide production (in nmol of O2/106 cells min−1; Figure 2A) was diminished in all G6PC3-deficient patients studied (patient A, 1.78; patient B, 1.54; patient C, 3.11) and in GSD-1b patients (patient X, 2.66; patient Y, 1.42) compared with the mean ± SEM (standard error of the mean) result in healthy controls (HCs: 7.17 ± 0.29; n = 5). Findings were similar in peripheral blood monocyte (PBMC)-derived macrophages (Figure 2B), where the production of hydrogen peroxide in response to phorbol-myristyl acetate (PMA) was diminished in patient cells compared with HCs. Neutrophil superoxide production in the parents of G6PC3-deficient patients (heterozygote carriers of the corresponding mutation in their offspring) was normal compared with HCs (6.07 ± 0.67, P = 0.10). This was confirmed by flow cytometry-based measurement of the (intracellular) respiratory burst (Figure 2C and D).

In the cell-free assay (Figure 2E), neutrophil membranes from patient A displayed comparable levels of oxidase activity in mol O2/s/mol cytochrome b558 (83.57 ± 5.94) to positive controls (77.03 ± 1.29), when reconstituted with recombinant cytosolic components of the NADPH oxidase.

Table I. Patient characteristics

<table>
<thead>
<tr>
<th>Pt</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Ethnic origin</th>
<th>Infections</th>
<th>Other phenotypic features</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>M</td>
<td>28</td>
<td>P</td>
<td>Bronchiectasis, Aspergillus pneumonia (11 years), recurrent bacterial pneumonia, PA</td>
<td>Short stature, type 2 ASD, IBD</td>
<td>c.[190_210]del + [190_210]del</td>
</tr>
<tr>
<td>B</td>
<td>F</td>
<td>16</td>
<td>P</td>
<td>Recurrent bacterial pneumonia, PA</td>
<td>Short stature, IBD</td>
<td>c.[190_210]del + [190_210]del</td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>20</td>
<td>P</td>
<td>Recurrent oral ulceration</td>
<td>—</td>
<td>c.[130C&gt;T] + [130C&gt;T]</td>
</tr>
<tr>
<td>D</td>
<td>M</td>
<td>6</td>
<td>T</td>
<td>Neonatal sepsis</td>
<td>Type 2 ASD, cryptorchidism, PSVP</td>
<td>c.[758G&gt;A] + [758G&gt;A]</td>
</tr>
<tr>
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<td>F</td>
<td>12</td>
<td>T</td>
<td>Pneumonia, sepsis</td>
<td>Type 2 ASD, PV stenosis, panniculitis, PSVP</td>
<td>c.[554T&gt;C] + [554T&gt;C]</td>
</tr>
</tbody>
</table>

M, male; F, female; P, Pakistani; T, Turkish; PA, perianal abscesses; ASD, atrial septal defect; PV, pulmonary valve; IBD, inflammatory bowel disease; PSVP, prominent superficial venous pattern; patients D and E correspond to patients 4 and 6, respectively, in Boztug et al. (2009).
Hexose-monophosphate shunt activity

The reduced neutrophil respiratory burst seen in GSD-1b is thought to arise as a result of impaired hexose-monophosphate shunt (HMPS) activity, which leads to a relative lack of NADPH as a substrate for the enzyme NADPH oxidase (Lange et al. 1980; Narisawa et al. 1986; Bashan et al. 1988; Potashnik et al. 1990; Kuijpers et al. 2003). A lack of NADPH is indicated by blunted HMPS activity in response to methylene blue. Although HMPS activity in G6PC3-deficient neutrophils was blunted in response to PMA, a normal response was observed after the addition of methylene blue (Figure 3). This response has also been described in chronic granulomatous disease (Holmes et al. 1967), where the dysfunction of the NADPH oxidase complex underlies the abnormal respiratory burst.

NADPH oxidase subunit analysis

Western blotting for NADPH oxidase components revealed an aberrant band for gp91phox with an abnormally low apparent molecular weight of ~65 kDa in all patients with G6PC3 mutations and in two unrelated patients with GSD-1b (Figure 4A and B). Expression of p67, p47 and p22phox in patient neutrophils was normal as was the cDNA sequence for the CYBB gene encoding gp91phox in patient A (data not shown). The latter data suggested that incomplete N-glycosylation was responsible for the abnormally low apparent molecular weight of gp91phox seen in patient neutrophils. Normal amounts of gp91phox were expressed on the cell surface in patients’ neutrophils, as determined by immunoreactivity on intact neutrophils (Figure 4C). In addition, reduced-minus-oxidized difference spectroscopy (Figure 4D) revealed a normal level of cytochrome b558 in patient (0.35 µM) compared with HC neutrophils (0.25 µM).

G6PC3 mutation induces major alterations in neutrophil N- and O-glycomes

The observation of an abnormally low molecular weight for gp91phox in patients indicated impaired glycosylation of this molecule. In order to determine whether global defects in neutrophil glycosylation are associated with G6PC3 mutations, we performed MS profiling of healthy and patient neutrophils.

The glycomes of the healthy neutrophil sample were in agreement with earlier studies on pooled neutrophils (Babu et al. 2009; see also data on the Consortium for Functional Glycomics website: www.functionalglycomics.org). Briefly, N-glycans included high mannose and complex bi-, tri- and tetra-antennary structures, of which the latter were core-fucosylated, nonbisected and terminated mainly with sialic acid and LewisX epitopes (Figure 5A). Agalactosylated N-glycan structures were found to be minor and restricted only to core-fucosylated bi- and tri-antennary structures (m/z 1835 and 2081; Figure 5A, upper panel). A great number of complex N-glycans were extended with N-acetyllactosamine (LacNAc) units, resulting in multibranched high-molecular-weight glycans (Figure 5A, lower panel). In contrast, neutrophil N-glycomes of patient A (Figure 5B) showed a dramatic reduction in high-molecular-weight glycans, which appears to be caused by a failure to incorporate galactose (Gal) into the majority of the complex-type glycans. Consequently, many of the complex glycans had truncated antennae. Of particular note is the dominant signal at m/z 2326 which is the molecular ion for the agalactosylated tetra-antennary N-glycan shown in the cartoon annotation. Other N-glycans deficient in Gal were observed at m/z 2530, 2704, 2891, 3095 and 3269 (Figure 5B). None of these were detected in the HC. The O-glycan profiles also exhibited defects in galactosylation, although to a lesser extent. Thus, the healthy O-glycome is comprised of sialylated core 1 (m/z 895 and 1256) and core 2 (m/z 983, 1344 and 1518) structures.
G6PC3 mutations are associated with a major defect of glycosylation

Figure 2. NADPH oxidase function is diminished in G6PC3-deficient phagocytes. (A) Superoxide production in neutrophils and (B) hydrogen peroxide production in macrophages after stimulation with PMA were measured in patients with G6PC3 mutations (G6PC3−/−), their parents (G6PC3+/−) and patients with GSD-1b and were compared with HCs (n = 5). (C) FACS analysis of the intracellular reduction in dihydrorhodamine by PMA-stimulated neutrophils detected two populations of cells at 15 min that had mounted a robust (N2) or weak (N1) respiratory burst compared with unstimulated cells (open histograms). (D) This result was used to calculate the “stimulation index” (Vowells et al. 1995) and expressed as a percentage of the mean HC response (n = 5). A–C and P1–P4 correspond to Figure 1 and Table 1. **P = 0.009 vs HCs. All experiments were conducted in triplicate and on at least two separate occasions. (E) Superoxide production in the cell-free assay (triplicate experiments) with membranes from patient A (filled bars) was comparable with that of positive controls (open bars; M, activity with membrane alone; M + C, reconstituted activity with membrane and cytosolic components).

Figure 3. HMPS activity in neutrophils. Neutrophils of Patient A (filled bars) were compared with HCs (n = 5; open bars). HMPS activity based on the liberation of 14CO2 from radiolabeled 14C1 glucose was diminished in response to PMA but normal after the addition of methylene blue. **P = 0.01; ns, P = 0.57; compared with HCs. All experiments were conducted in triplicate and on at least two separate occasions.

(Figure 5C), whereas the patient lacks Gal on the core 2 antenna (m/z 1140; Figure 5D), but retains normal galactosylation of core 1 sequences (m/z 895 and 1256). Taken together, these data suggest that the G6PC3 mutation induced a profound alteration on the N- and O-glycosylation profiles of patient’s neutrophils, characterized by the almost complete absence of tetra-antennary and LacNAc extended N-glycans and mature core 2 O-glycans. Similarly, abnormal glycomic profiles were also demonstrated in patient B (the sibling of patient A), an unrelated patient with G6PC3 mutation (patient D) and a patient with GSD-1b (patient Y; Figure 6). The absence of glycans with mature antennae in the glycomic profiles was fully compensated for by the presence of abundant peaks for glycans with truncated antennae. Also, the overall quality of the glycomic data obtained from the patient’s neutrophils was in accord with the normal levels of N- and O-glycan occupancy on their glycoproteins.

ER stress-related protein expression in neutrophils

Expression of the ER stress-related proteins Grp78 and pEIF2α was increased both in patient A with G6PC3 deficiency and in an unrelated patient (Y) with GSD-1a (Figure 7).

Discussion

In this study, we have made the remarkable discovery that both G6PC3 mutations and the GSD-1b syndrome are associated with a major defect of neutrophil glycosylation. Thus, glycomic analysis of patient neutrophils showed truncated glycosylation of the majority of their complex-type N-glycans, as well as the core 2 antennae of their O-glycans. Glycans were arrested at an "immature" stage and were profoundly deficient in the sialylated and LewisX epitopes, characteristic of the normal neutrophil glycome (Haslam et al. 2006; Babu et al. 2009). The antennae of the abnormal N-glycans were observed as GlcNAc "stubs", indicating a failure of galactosylation. We hypothesize that this could be the result of insufficient levels of uridine diphosphate (UDP)-Gal in the Golgi apparatus. The formation of complex N-glycans involves the strict, stepwise addition of sugar residues (Spiro 2002), and a lack of UDP-Gal would lead to truncation at the stage observed in our patients. Such truncation would also result in
a reduction in glycan-binding protein epitopes for functionally important lectins such as selectins, siglecs and galectins with profound implications for cellular function. Hypo-galactosylation of the O-glycans of the patients’ neutrophils was observed on their core 2 antennae. In contrast, galactosylation to form the core 1 precursor was found to be normal, implying that UDP-Gal levels are sufficient for the first galactosylation step in O-glycan biosynthesis but not for events in later Golgi compartments. However, our data do not exclude the possibility that entire glycan chains are absent from NADPH oxidase or other glycoproteins. Complex ER–Golgi interactions may underlie what appear to be partial and selective absence of Gal residues. The underlying biochemical causes of hypo-galactosylation remain enigmatic, and further work is needed to establish how impaired hydrolysis of G6P affects Gal metabolism. Interestingly, we observe the normal levels of capping with sialic acid or fucose of the small minority of glycans whose antennae had been successfully galactosylated, suggesting that the metabolism of these sugars is not impaired.

The electron-transporting subunit of NADPH oxidase, gp91phox, has a 58 kDa (unglycosylated) “protein backbone” (Kleinberg et al. 1989) and is N-glycosylated at Asn-132, -149 and -240 (Wallach and Segal 1997; Taylor et al. 2006). In neutrophil lysates from all five patients with G6PC3 mutations and the two patients with GSD-1b, western blots for gp91phox demonstrated that much of this glycoprotein had an apparent mass of ~65 kDa, rather than the ~90 kDa exhibited by normal gp91phox. The latter has been reported to carry complex N-glycans at three sites (Harper et al. 1985; Taylor et al. 2006) and its apparent molecular weight is ~15 kDa higher than predicted for typical neutrophil N-glycosylation. However, this is not surprising, as it is well known that highly glycosylated proteins exhibit anomalous SDS–PAGE behavior (Segrest et al. 1971). On the other hand, the observed mass difference of ~7 kDa between the patients’ gp91phox and the unglycosylated backbone of gp91phox is fully consistent with the occupancy of all three glycosylation sites with the abnormal glycans observed in our glycomic analysis of the patients’ neutrophils.

The diminished neutrophil respiratory burst seen in GSD-1b is thought to be due to a lack of NADPH as a consequence of attenuated HMPS activity (Chou et al. 2002). However, the HMPS is a cytosolic cascade (Wamelink et al. 2008) dependent on the supply of glucose-1-phosphate which, in neutrophils, is primarily derived from stored glycogen (Stossel et al. 1970)—a process which would not appear to be dependent on glucose transport and metabolism in the ER. The rate-limiting enzyme of the HMPS, G6P dehydrogenase, is allosterically induced by NADP and inhibited by NADPH. In this way, normal activity of the NADPH oxidase in neutrophils stimulates the HMPS and in the presence of oxidase dysfunction, it is suppressed by accumulating NADPH. In the latter situation, HMPS activity may be stimulated by another means of NADPH consumption. We demonstrated that HMPS activity was diminished in G6PC3-deficient neutrophils in response to PMA (an activator of the NADPH oxidase), but normal in response to methylene blue—an oxidizing agent
which leads to the consumption of NADPH (Holmes et al. 1967). In patients bearing a G6PC3 mutation, therefore, there does not appear to be a lack of NADPH. Dysfunction of the NADPH oxidase enzyme complex could therefore be responsible for the abnormal respiratory burst seen in these cells. The observation of aberrant gp91phox in patients with GSD-1b raises the possibility that it may also contribute to the diminished respiratory burst seen in this condition.

The exact mechanism of this dysfunction is unclear. The cell-free assay results indicate that, at least in the artificial conditions achieved in the presence of lithium dodecyl sulfate (LiDS), NADPH oxidase activity in patients is comparable to positive control. Abnormal glycosylation does not, therefore, appear to interfere with electron transport or assembly of the oxidase complex in the cell-free assay. The latter is in accordance with a previous study showing that heterodimer formation (binding) of gp65–p22phox, gp65 being the biosynthetic precursor of gp91phox exiting the ER (Yu et al. 1999), does not require glycosylation (DeLeo et al. 2000). An alternative hypothesis (Wallach and Segal 1997), which has been established for other glycoproteins (Da Silva and Gordon 1999; Qureshi et al. 2007), is that N-glycosylation may serve to protect gp91phox from the contents of the phagocytic vacuole. This could account for a diminished respiratory burst seen in G6PC3-deficient cells.

The neutropenia observed in GSD-1b is attributed to premature apoptosis. Again thought secondary to a lack of NADPH (Kuijpers et al. 2003; Leuzzi et al. 2003; Kardon et al. 2008), GSD-1b neutrophils would have a diminished capacity to (re-) generate reduced glutathione. Cells would thus be rendered more susceptible to oxidative damage and undergo apoptosis as a result. Increased spontaneous apoptosis has also been observed in G6PC3-deficient neutrophils (Boztug et al. 2009). However, given that there does not appear to be a lack of NADPH in these cells, an alternative pathway must be proposed. ER stress and the induction of

**Fig. 5.** G6PC3 mutation is associated with incomplete glycosylation in neutrophil N- and O-glycomes. MALDI-TOF mass spectra of permethylated N- and O-glycans derived from control (A and C) and patient A (B and D) neutrophils. (A and B) Upper panels depict low mass spectra and lower panels depict high mass spectra of permethylated N-glycans. Peaks designated with LacNAc units (m/z 3415, 3677, 3851, 3864, 4038, 4127, 4301, 4313, 4488, 4576, 4750, 4763 and 4937) correspond to annotated N-glycan structures extended with LacNAc repeats. (B) Annotated peaks in red correspond to N-glycan structures not found in (A), while nonannotated peaks denote the same structures as in (A). (D) Annotated peaks in red correspond to O-glycan structures significantly increased relatively to (C), while non-annotated peaks denote the same structures as in (C). Structures are according to the Consortium for Functional Glycomics (www.functionalglycomics.org) guidelines and were deduced from monosaccharide composition, MALDI-TOF/TOF MS/MS data and knowledge of biosynthetic pathways. All molecular ions are [M + Na]+. Structures that show sugars outside a bracket have not been unequivocally defined.
GSK3β have been documented in G6PC3-deficient neutrophils (Boztug et al. 2009). The finding of increased levels of ER stress-related proteins in both G6PC3-deficient and GSD-1b neutrophils (Figure 7) suggests that this phenomenon may have a role to play under both conditions.

In summary, our findings provide a novel mechanism for the neutrophil dysfunction seen in both G6PC3 mutation and GSD-1b in that both exhibit profound hypo-galactosylation of N- and O-glycans. The observed defects in glycosylation merit the designation of congenital disorders of glycosylation (CDGs) to both syndromes. It should be noted that the classic diagnostic blood test for CDGs, which involves checking the glycosylation status of serum glycoproteins such as transferrin (Freeze and Aebi 2005), will not diagnose the G6PC3 syndrome. This is because marker glycoproteins in the serum are largely liver derived and this organ uses G6PC1 instead of G6PC3 for G6P metabolism. Therefore, a cellular or a more specialized test will be required. Finally, it is important to bear in mind that the ubiquitous expression of G6PC3 suggests that glycosylation defects are unlikely to be restricted

Fig. 6. Neutrophil N- and O-glycomes from patients B, D and Y. Presentation of results and annotation used in these figures are identical to that in Figure 5. Similar patterns of truncated glycosylation to those seen in patient A were observed in patient B (A and B) and patient D (C and D), with G6PC3-deficiency. Patient Y (E and F) with GSD-1b, displayed a similar pattern of truncated glycosylation.
The respiratory burst was studied in neutrophils and macrophages. First, $2 \times 10^6$ neutrophils were stimulated with 1 µg mL$^{-1}$ PMA (Sigma) in a temperature-controlled (37°C) spectrophotometer cuvette assay based on the reduction in cytochrome c (Sigma). The color change produced by superoxide dismutase-inhibitable cytochrome c reduction was detected at 550 nm and the maximal rate of change in absorbance was compared with a control cuvette containing cells and cytochrome c without the addition of PMA. The maximal rate of change in absorbance (nm min$^{-1}$) was converted to nmol O$_2$/10$^6$ cells min$^{-1}$ using a previously published formula (Dahlgren and Karlsson 1999). Triplicated experiments, again based on the reduction in cytochrome c (Teufelhofer et al. 2003), were conducted with neutrophils using a temperature-controlled (37°C) microplate reader (Omega FLUOStar, BMG Labtech, UK). Second, the intracellular reduction in dihydrodihydrocarbamine (Sigma) was measured in response to 1 µg mL$^{-1}$ PMA by fluorescence-activated cell sorter (FACS) analysis, using a modification of a previously published method where purified neutrophils were used (as opposed to whole blood samples; Vowells et al. 1995). Mean fluorescence in the gated populations of cells indicated (N2) was used to calculate the “stimulation index” as a ratio of the result produced by unstimulated cells in paired reactions. For patients, this was expressed as a percentage of the mean response from a study of five HC volunteers. The macrophage respiratory burst was studied in a microplate assay (Rahman et al. 2009) in response to PMA using the Ampex Red reagent (Invitrogen) to produce a color change as the consequence of production of hydrogen peroxide.

**Cell-free assay (Molshanski-Mor et al. 2007)**

Neutrophil membranes were purified from whole blood lysates, in the presence of protease inhibitors, by gradient centrifugation and diluted to achieve 1 pmol of cytochrome b$_{558}$ per well. NADPH oxidase activity was measured by cytochrome c reduction in a 96-well plate format from wells containing membrane alone or after the addition of the cytosolic components of the NADPH oxidase (all purified human recombinant proteins): p47$^{phox}$ (100 nM), p67$^{phox}$ (100 nM) and Rac1 (Q61L)-GTP (100 nM). Incubation with 130 µmol of LiDS was carried out for 90 s prior to the addition of 238 µM NADPH. Positive control was obtained from solubilized guinea-pig macrophage membranes.

**Materials and Methods**

**Participants**

Heparinized (6 U mL$^{-1}$) blood samples were obtained from patients, relatives and controls with informed consent with approval from the joint UCL/UCLH committees on the ethics of human research. Patient details are summarized in Table I. The fraternal twin of patient A has sensorineural deafness but otherwise normal phenotype. Cell samples from patients D and E were kindly provided by K.B. and C.K. and from patient C by R.K.G. Patients with GSD-1b were unrelated, with previously characterized mutations in SLC37A4 (performed by the Sheffield Molecular Genetics Service, UK; Veiga-da-Cunha et al. 1998). All patients were sampled after treatment with granulocyte colony-stimulating factor had been commenced. The attenuated respiratory burst in patient A was unaffected by treatment (data not shown), but peripheral blood neutrophil counts corrected to normal range.

**Macrophage culture and neutrophil isolation**

PBMCs were isolated from whole blood by centrifugation through Lymphoprep® (Axis Shield, Norway) and cultured for 5 days in an RPMI-1640 medium (Gibco, UK) and supplemented with 10% fetal calf serum (Sigma, UK) with 100 U mL$^{-1}$ penicillin and 100 µg mL$^{-1}$ streptomycin (Gibco). Adherent cells were regarded as differentiated as described previously (Smith et al. 2009). After the removal of monocytes from the above centrifugation, neutrophils were isolated by sedimentation with 1% dextran and hypotonic lysis of red blood cells. Cells were counted and re-suspended in phosphate-buffered saline (PBS; Gibco) for all subsequent investigations.

**Respiratory burst assays**

The respiratory burst was studied in neutrophils and macrophages. First, $2 \times 10^6$ neutrophils were stimulated with 1 µg mL$^{-1}$ PMA (Sigma) in a temperature-controlled (37°C) spectrophotometer cuvette assay based on the reduction in cytochrome c (Sigma). The color change produced by superoxide dismutase-inhibitable cytochrome c reduction was detected at 550 nm and the maximal rate of change in absorbance was compared with a control cuvette containing cells and cytochrome c without the addition of PMA. The maximal rate of change in absorbance (nm min$^{-1}$) was converted to nmol O$_2$/10$^6$ cells min$^{-1}$ using a previously published formula (Dahlgren and Karlsson 1999). Triplicated experiments, again based on the reduction in cytochrome c (Teufelhofer et al. 2003), were conducted with neutrophils using a temperature-controlled (37°C) microplate reader (Omega FLUOStar, BMG Labtech, UK). Second, the intracellular reduction in dihydrodihydrocarbamine (Sigma) was measured in response to 1 µg mL$^{-1}$ PMA by fluorescence-activated cell sorter (FACS) analysis, using a modification of a previously published method where purified neutrophils were used (as opposed to whole blood samples; Vowells et al. 1995). Mean fluorescence in the gated populations of cells indicated (N2) was used to calculate the “stimulation index” as a ratio of the result produced by unstimulated cells in paired reactions. For patients, this was expressed as a percentage of the mean response from a study of five HC volunteers. The macrophage respiratory burst was studied in a microplate assay (Rahman et al. 2009) in response to PMA using the Ampex Red reagent (Invitrogen) to produce a color change as the consequence of production of hydrogen peroxide.

**Cell-free assay (Molshanski-Mor et al. 2007)**

Neutrophil membranes were purified from whole blood lysates, in the presence of protease inhibitors, by gradient centrifugation and diluted to achieve 1 pmol of cytochrome b$_{558}$ per well. NADPH oxidase activity was measured by cytochrome c reduction in a 96-well plate format from wells containing membrane alone or after the addition of the cytosolic components of the NADPH oxidase (all purified human recombinant proteins): p47$^{phox}$ (100 nM), p67$^{phox}$ (100 nM) and Rac1 (Q61L)-GTP (100 nM). Incubation with 130 µmol of LiDS was carried out for 90 s prior to the addition of 238 µM NADPH. Positive control was obtained from solubilized guinea-pig macrophage membranes.

**HMPS activity**

About $1 \times 10^6$ neutrophils were stimulated with either 1 µg mL$^{-1}$ PMA or 300 µM methylene blue (BDH Pharmaceuticals), in a modification of a previously published method (Holmes et al. 1967). Briefly, reactions were conducted in sealed containers in 1 mL of PBS containing 1 mM glucose and 0.5 µCi (0.00185 Mbo) $^{14}$C glucose (Perkin-Elmer). $^{14}$CO$_2$ released by the activity of the HMPS (Wamelink et al. 2008) was collected onto a filter paper saturated with 20% sodium hydroxide and suspended above the reaction mixture within the closed container. After 1 h, the filter paper was removed into 5 mL scintillation fluid (Ultima Gold®, Perkin Elmer) and counted immediately in a scintillation counter calibrated for $^{14}$C (Tricarb 2100TR, Perkin Elmer). Results thus obtained were expressed as counts per minute. All experiments were repeated on at least twice.

“Protein lysates” were prepared using a previously published method (Alterman et al. 1990), and 20 µg of protein per lane was separated using SDS–PAGE (8% gel for gp91 and p67, and 10% for p47 and p22). Protein lysates from other patients with G6PC3 mutations were also kindly supplied by C.K. MoAb48 (gift from Prof Dirk Roos,
Amsterdam, the Netherlands) was used to detect gp91, whereas other NADPH oxidase subunit antibodies were prepared “in-house”. Antibodies to Grp78, pEiF2α and actin (Abcam) were used according to the manufacturer’s protocols.

Surface expression of gp91phox and spectral analysis

Purified neutrophils were suspended in PBS (Gibco) containing 0.1% bovine serum albumin (Merck) and 0.01% sodium azide (Sigma). FACS analysis for gp91phox was performed using a commercially available fluorescein isothiocyanate-conjugated antibody (7D5; MBL International). The reduced-minus-oxidized UV/visible difference spectra of intact neutrophils were recorded 10 min after the addition of sodium dithionite to 3 × 107 cells stimulated with 1 µg mL−1 PMA, in PBS at pH 7.3 and at 37°C. The concentration of cytochrome b558 was calculated from the difference between the height of the 559 nm peak and the depth at 540 nm, using an extinction coefficient of ε559 = 21.6 mM⁻¹ cm⁻¹ (Roberts et al. 1982).

Glycomic profile analysis

Snap-frozen neutrophils (10⁷) purified as above were treated as described previously (Jang-Lee et al. 2006; Sutton-Smith and Dell 2006). Briefly, all samples were subjected to homogenization using a 130 W Vibra-Cell ultrasonic processor (VC 130 PB, Sonics & Materials) within a sound-abating enclosure in an extraction buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA and 1% (3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate, at pH 7.4), reduction in 4 M guanidine–HCl (Pierce), carboxymethylation and trypsin digestion. The digested glycoproteins were purified by C18-Sep-Pak (Waters Corp). N-Glycans were released by PNGaseF (EC 3.5.1.52, Roche Applied Science) digestion, whereas O-glycans were released by reductive elimination. N- and O-glycans were then permethylated using the sodium hydroxide procedure, and finally, the permethylated N- and O-glycans were purified by C18-Sep-Pak.

All permethylated samples were dissolved in 10 µL of methanol and 1 µL of dissolved sample was premixed with 1 µL of matrix (20 mg mL⁻¹ 2,5-dihydroxybenzoic acid in 70%, v/v, aqueous methanol), spotted onto a target plate (2 × 1 cm²) and dried under vacuum. MS data were acquired using a Voyager-DE STR matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF; Applied Biosystems). MS/MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. The collision energy was set to 1 kV, and argon was used as a collision gas. The 4700 Calibration Standard Kit, Calmix (Applied Biosystems), was used as the external calibrant for the MS mode of both instruments, and [Glu1] fibronectopeptide B human (Sigma) was used as an external calibrant for the MS/MS mode of the MALDI-TOF/TOF instrument.

The MS and MS/MS data were processed using Data Explorer 4.9 Software (Applied Biosystems). The spectra were subjected to manual assignment and annotation with the aid of the glycobioinformatics tool GlycoWorkBench (Ceroni et al. 2008). The proposed assignments for the selected peaks were based on ¹³C isotopic composition together with knowledge of the biosynthetic pathways. The proposed structures were then confirmed by data obtained from MS/MS experiments.

Mutation analysis

DNA was extracted from patient’s peripheral blood leucocytes using standard procedures. All patients were screened for mutations in ELA2, HAX1 and WAS (none identified). All six coding exons and flanking introns of the G6PC3 gene (RefSeq NM_138387) were amplified via the polymerase chain reaction (PCR) using the high-fidelity enzyme Phusion (New England Biolabs) according to manufacturer’s instructions. Primer sequences are available on request. PCR products were screened for mutations by denaturing high-performance liquid chromatography using the WAVE platform (Transgenomic). Homozygous G6PC3 mutations were identified by mixing equal amounts of patient PCR product with a control PCR product known to be homozygous wild type, prior to denaturation to allow the heteroduplex formation. Patient samples with abnormal WAVE chromatograms were sequenced using the DTCS Quick Start Kit and CEQ8000 DNA Genetic Analysis System (Beckman Coulter). Effects on protein sequence were predicted using Ensembl release 55 (©Wellcome Trust Sanger Institute/European Bioinformatics Institute).

Statistical analysis

Error bars in all figures represent the SEM. Comparisons (using Prism v4.0, GraphPad) were made between groups using Student’s t-test if data were normally distributed (by Shapiro-Wilk normality test) or two-tailed Mann–Whitney U-test if not. Significance was assumed at P < 0.05.

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Conflict of interest

None declared.

Abbreviations

CDG, congenital disorder of glycosylation; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; G6P, glucose-6-phosphate; G6PC, glucose-6-phosphatase catalobic; G6PT1, glucose-6-phosphate translocate-1; GSD-1a, glycogen storage disease type-1a; HC, healthy control; HMPS, hexose-monophosphate shunt; LacNac, N-acetyllactosamine; LiDS, lithium dodecyl sulfate; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight mass spectrometer; MS, mass spectrometric; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PBMC, peripheral blood monocyte; PCR, polymerase chain reaction; PMA, phorbol-myristyl acetate; PNGaseF,
peptide-N-glycosidase F; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEM, standard error of the mean; UDP, uridine diphosphate.

References


G6P/C3 mutations are associated with a major defect of glycosylation.


