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Plasmodium falciparum infection induces dynamic changes in the erythrocyte phospho-proteome

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ABSTRACT

The phosphorylation status of red blood cell proteins is strongly altered during the infection by the malaria parasite *Plasmodium falciparum*. We identify the key phosphorylation events that occur in the erythrocyte membrane and cytoskeleton during infection, by a comparative analysis of global phospho-proteome screens between infected (obtained at schizont stage) and uninfected RBCs. The meta-analysis of reported mass spectrometry studies revealed a novel compendium of 495 phosphorylation sites in 182 human proteins with regulatory roles in red cell morphology and stability, with about 25% of these sites specific to infected cells. A phosphorylation motif analysis detected 7 unique motifs that were largely mapped to kinase consensus sequences of casein kinase II and of protein kinase A/protein kinase C. This analysis highlighted prominent roles for PKA/PKC involving 78 phosphorylation sites. We then compared the phosphorylation status of PKA (PKC) specific sites in adducin, dematin, Band 3 and GLUT-1 in uninfected RBC stimulated or not by cAMP to their phosphorylation status in iRBC. We showed cAMP-induced phosphorylation of adducin S59 by immunoblotting and we were able to demonstrate parasite-induced phosphorylation for adducin S726, Band 3 and GLUT-1, corroborating the protein phosphorylation status in our erythrocyte phosphorylation site compendium.

1. Introduction

Mammalian erythrocytes have been widely studied for cytoskeleton structure, membrane composition and transport properties. Over the last decade, numerous studies have incorporated mass spectrometry techniques to mine and quantify the proteins expressed in red blood cells (RBCs) and to link these proteins functionally to various physiological or pathophysiological situations. However, even if these cells have a simple structural organization and specific function, proteomics studies have proven difficult to get a good overview of the diversity of proteins in RBCs for several reasons: (i) the abundance of ultra-majoritarian proteins in the cytosol (haemoglobin, up to 98% of cytosolic proteins at an unrivalled concentration: 5 mM–340 g/l) and in the membrane (Band 3, 1 M copies/cell) and (ii) the tight links between membrane and sub-membranous cytoskeleton. With the progression of techniques, the number of proteins identified has raised to an unexpected number of 1578 unique proteins in the cytosol [1], and a total number of 2289 unique proteins in the RBC identified so far [2].

The next challenge now is to link these proteins to physiological processes, and one key feature is to elucidate the regulatory role of various post-translational modifications (PTMs). These modifications include oxidation effects, glycosylation, palmitoylation and most of all protein phosphorylation. Indeed, kinase activities have been described as a key regulatory mechanism in RBC and deregulation of their activities seems to be implicated in multiple diseases [3] including malaria, the focus of this study. Human RBCs in circulation harbour various active protein kinases, including protein kinase C (PKC), protein kinase A (PKA), casein kinases I and II (CK1 and CK2), Syk, Lyn, Hck–Fgr, and Fyn, as reviewed by Pantaleo et al. [4]. Activity of these kinases has been mostly studied regarding their effects on cytoskeleton or membrane proteins in various physiological or pathophysiological contexts.

Spectrin phosphorylation by casein kinase has been associated with membrane destabilization [5] and enhanced spectrin phosphorylation is linked to hereditary elliptocytosis and pyropoikilocytosis [6]. The horizontal junctional complex components are also phosphorylated; the combined phosphorylation of adducin and protein 4.1 by PKC decreased

Abbreviations: CK1 and CK2, casein kinases I and II; GO, gene ontology; GLUT-1, glucose transporter 1; iRBC, infected red blood cell; LC–MS/MS, liquid chromatography tandem mass spectrometry; LFQ, label free quantification; PTMs, post-translational modifications; PKA, protein kinase A; PKC, protein kinase C.

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their binding to spectrin and actin, resulting in weaker membrane stability [7]; dematin is known to be phosphorylated by PKA, leading to disruption of actin/spectrin binding [8]. Regarding vertical complexes and integral membrane proteins, Band 3 PTMs have been widely studied (in regard to its pivotal role in membrane transport and structure). Notably, Band 3 tyrosine phosphorylation triggers its dissociation from ankyrin, consequently releasing Band 3 from spectrin/actin cytoskeleton [9].

The present study focusses on the changes in RBC protein phosphorylation resulting from infection with the human malaria causing parasite *Plasmodium falciparum*. During its complex life cycle, the parasite invades RBCs, where it is largely hidden from the host immune system. Inside erythrocytes it multiplies via the process of schizogony every 48 h to form up to 32 new merozoites. After rupture of infected RBCs, merozoites are released to invade fresh red cells and to complete a new cycle of asexual development. During intraerythrocytic development, the parasite remodels the red blood cell surface, notably by exporting proteins to the host cytosol and membrane and by forming knobs that mediate interactions with host endothelial cells to escape clearance of infected RBCs (iRBC) by the spleen. The process of sequestration and cytoadherence of iRBCs results in clogging of blood vessels in various organs and it contributes to the clinical symptoms of malaria. Host cell permeability [10] or deformability [11] are also largely modified upon infection. Various exported parasite proteins play crucial roles in this process, and lately it's becoming clear that an involvement of host proteins is also required for parasite-mediated host cell remodelling. Multiple host proteins see their phosphorylation status altered during infection, which was demonstrated by the pioneering study of Wu et al. [12], who in 2009 showed increased phosphorylation during parasite infection by immunoblotting and mass spectrometry. With the emergence of liquid chromatography tandem mass spectrometry, several studies [13–16] followed with phospho-proteome analyses of iRBCs, but remarkably largely ignored published data on phosphorylation sites identified in human red blood cell proteins. This study summarizes our current knowledge on RBC phosphorylation by comparing published phosphorylation sites measured in normal and *P. falciparum*-infected RBCs and experimentally validating some of the major findings.

2. Material and methods

2.1. Compendium of red blood cell phosphorylation sites

Phospho-peptide sequences identified in large scale RBC phosphoproteome LC–MS/MS studies with data taken from normal [17] and *P. falciparum*-infected RBCs at the schizont life cycle stage [13–16] were remapped to the UNIPROT protein database using the software tool Protein Coverage Summarizer (http://omics.pnl.gov/software/protein-coverage-summarizer) in order to generate a uniform format for comparing phosphorylation sites across data sets. The compendium of phospho-sites included all sites detected in normal RBCs from Soderblom et al. [17], and sites from infected RBCs that were detected at least twice in the four independent studies [13–16].

2.2. Phosphorylation motif analyses

Phosphorylation sites were categorized by their chemical properties as acidic, basic, proline-directed, tyrosine or other by a decision tree method described previously [14] as follows: 1) get the 6 neighbouring amino acids before and after the phosphorylation site; 2) pY at position 0 then classify as "Tyrosine"; 3) P at +1 then classify as "Proline-directed"; 4) positions +1 to +6 contain more than one D and E residues then classify as "Acidic"; 5) K or R at position −3 then classify as "Basic"; 6) D or E at +1, +2, or +3 then classify as "Acidic"; 7) between −6 and −1 more than 2 K or R residues then classify as "Basic"; 8) remaining peptides classify as "Other". Phosphorylation motifs were identified using MotifX [18] with the following parameters: phosphorylation motif window = 13 amino acids, p-value threshold = $1 * 10^{-4}$ for S and T residues, $1 * 10^{-3}$ for Y residues, motif fold increase ≥2, a motif frequency >5, and a background of all RBC proteins identified. The analysis was repeated for a degenerate amino acid set with conservative amino acid substitutions within the motif window according to: A = AG, D = DE, F = FY, K = KR, I = ILVM, Q = QN, S = ST, C = C, H = H, P = P, W = W. When different motifs were found for a peptide by the analyses with different amino acid residues, priority was given to the motif with the highest MotifX score. Sequence logos were generated with Weblogo 3 [19] from http://weblogo.threeplusone.com/create.cgi. The motifs were matched to known protein kinase target motifs using PhosphoMotifFinder [20] and matches were considered as potential links between phosphorylation motifs and protein kinases.

2.3. Gene ontology (GO) analysis

GO enrichment analyses of lists of membrane-associated phosphoproteins from infected and normal RBCs were carried out using the web tool Database for Annotation Visualization and Integrated Discovery (DAVID, version 6.7. http://david.abcc.ncifcrf.gov/) [21,22] with a background set of all human proteins. Enrichment of GO FAT terms was considered statistically significant when corrected for multiple testing by the Benjamini–Hochberg method with adjusted p-values lower than 0.05. Overlap between enriched GO terms was visualised in a network with the Cytoscape plugin Enrichment Map [23].

2.4. Cell preparation and Western blotting

Human RBCs were drawn from healthy volunteers under informed consent, washed several times in RPMI, kept in culture medium (see below) and used within one week of collection. *P. falciparum* NF54 was grown in RBCs as described previously, using 0.5% Albumax II® (Invitrogen) instead of human serum [52]. Parasites were synchronized twice using 5% *w/v* sorbitol solution according to Lambros and Vanderberg [53]. Schizont-stage parasite cultures were then enriched to >95% by centrifugation on a 70% Percoll/sorbitol solution as described in [24]. Parasite growth and development of schizont stages was monitored by Giemsa-stained thin blood smears. Uninfected RBC controls were incubated for 48 h in culture medium at 37 °C and kept in the presence or absence of 50 μM dibutyryl-cAMP (Sigma Aldrich), a cell-permeable non-hydrolysable cAMP analogue that activates protein kinase A, for the last 30 min before harvesting. Cell counts were determined by using a Cellometer Mini (Nexcelom Bioscience) automated cell counter following manufacturer's recommendations.

Western blotting of uninfected RBCs and *P. falciparum*-infected RBC (1×10^7) samples was performed using erythrocyte ghosts. Briefly, RPMI-washed erythrocytes were incubated in 20 volumes of ice-cold hypotonic buffer 5P8 (5 mM NaH_2PO_4, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mix (1 μg each of chymostatin, leupeptin, antipain, and pepstatin and 8 μg aprotinin per ml), and phosphatase inhibitors (10 mM Na fluoride, 2 mM β-D glycerophosphate, 1 mM Na orthovanadate), then washed several times in 5P8 containing protease and phosphatase inhibitors (30 min centrifugation 14,000 ×*g* at 4 °C). Membrane proteins were solubilized in lysis buffer (150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 20 mM Tris–HCl pH 7.5, 1% Triton X-100) in the presence of protease and phosphatase inhibitors. *P. falciparum*-infected RBC samples were further lysed by sonication followed by centrifugation at 10,000 ×*g*. Dephosphorylation of proteins was achieved by incubation with λ-protein phosphatase in 1× NEBuffer (New England BioLabs, Inc.) supplemented with 1 mM $MnCl_2$ for 30 min at 30 °C. A Bradford protein assay kit (Bio-Rad Laboratories) was used to determine protein concentrations. Protein samples diluted in reducing Laemmli buffer