Deep Coverage Mouse Red Blood Cell Proteome

A FIRST COMPARISON WITH THE HUMAN RED BLOOD CELL

Erica M. Pasini, Morten Kirkeegaard, Doris Salerno, Matthias Mann, and Alan W. Thomas

Molecules have close genetic/physiological relationships to humans, breed rapidly, and can be genetically modified, making them the most used mammal in biomedical research. Because the red blood cell (RBC) is the sole gas transporter in vertebrates, diseases of the RBC are frequently severe; much research has therefore focused on RBC and cardiovascular disorders of mouse and humans. RBCs also host malaria parasites. Recently we presented an in-depth proteome for the human RBC. Here we present directly comparable data for the mouse RBC as membrane only, soluble only, and combined membrane bound/soluble proteomes (comprising, respectively, 247, 232, and 165 proteins). All proteins were identified, validated, and categorized in terms of subcellular localization, protein family, and function, and in comparison with the human RBC, were classified as orthologs, family-related, or unique. Splice isoforms were identified, and polypeptides migrating with anomalous apparent molecular weights were grouped into putatively ubiquitinated or partially degraded complexes. Overall there was close concordance between mouse and human proteomes, confirming the unexpected RBC complexity. Several novel findings in the human proteome have been confirmed here. This comparison sheds light on several open issues in RBC biology and provides a departure point for more comprehensive understanding of RBC function. *Molecular & Cellular Proteomics* 7: 1317–1330, 2008.

Animal research has been indispensable in understanding human biology and disease, and the most commonly used model has been the mouse (*Mus musculus*). There are now thousands of inbred strains and genetically modified lines available, and some, such as the C57BL/6 strain, have been propagated for almost 100 years. The C57BL/6 strain was chosen for the first mouse genome sequence (1) and for that reason has been used here. Mice share many characteristics with humans, and many of the strains and lines develop or are susceptible to diseases of the immune, endocrine, nervous, cardiovascular, and skeletal systems that have important similarities to human disease. The red blood cell (RBC) is one of the most important cell populations in any mammalian organism and has therefore been an object of intensive investigation for many years. Mouse RBCs have served as models for study of hemolysis (2, 3), infectious diseases (4–8), and different forms of anemia (9–13) since the early 1950s. In the sixties and seventies, the establishment of routine in vitro bone marrow cultures (14) opened investigation of hematopoiesis (15, 16), blood cell differentiation (17–19), and abnormalities thereof (19, 20). At the same time, interest developed in components of the immune system (21–23), cancer-related phenomena such as erythroleukemia (24–26), and the use of irradiated mouse models to explore the role of RBCs and other blood components in transplantation (27, 28). Continuous improvement in biochemical, imaging, and genetic techniques has ensured that the mouse remains the most used biomedical animal model. The availability of high accuracy and high sensitivity mass spectrometry techniques offers, for the first time, the prospect of deriving in-depth proteomes for critical cell types. Because of the experimental importance of the mouse we felt it critical to undertake a comparison of a deep coverage mouse RBC proteome with the recently derived deep coverage human (29) RBC proteome. To our knowledge this is not only the first global analysis of mouse membrane and soluble RBC proteins but also the first comparative RBC proteomics analysis in which samples were prepared and analyzed under identical conditions and data were validated according to consistent criteria.

The abbreviations used are: RBC, red blood cell; WBC, white blood cell; LTO-FT, Thermo Electron hybrid two-dimensional linear quadrupole ion trap-FTICR mass spectrometer; QSTAR, PE Sciex QSTAR Pulsar quadrupole orthogonal time-of-flight mass spectrometer; IPI, International Protein Index; EBI, European Institute for Bioinformatics; BLAST, Basic Local Alignment Search Tool; MM, mouse membrane; MM&S, mouse membrane and soluble; HS, human soluble; HM, human membrane; Hsp, heat shock protein; NOS, nitric-oxide synthase; eNOS, endothelial NOS.
Mouse RBC Proteome: a Comparison with the Human RBC

MATERIALS AND METHODS

RBC Preparation—Whole blood of adult C57BL/6 mice was collected in citrate either by saphenous vein puncture (200 locally anesthetized (Marcaine®) living mice, 0.2–0.3 ml/mouse) or by cardiac puncture immediately after administration of a deadly dose of isoflu- rane. To remove stress-related effects mice had been acclimated to the restraining conditions. All aspects of the work had received prior approval from the Biomedical Primate Research Centre animal experimental committee (Dier Experimenten Commissie). Saphenous and cardiac samples were processed separately at 4°C. White cells were removed (Plasmodont®; Euro-Diagnostica B.V., Arnhem, The Netherlands) according to the manufacturer’s instructions before RBCs were pelleted (1700 × g for 5 min), resuspended, and layered on Lympholyte-M cell separation medium (Lymphoprep, Axis-Shield, Oslo, Norway) (1700 × g for 5 min) to remove granulocytes and residual lymphocytes; at each step, along with supernatant, the upper RBC layer was removed. As for our previous study of human RBCs (29) samples were stored before analysis (4°C for 72–96 h) to allow maturation of reticulocytes to RBCs. Because, compared with humans, mouse blood has a high proportion of reticulocytes and a high platelet mass a further step was used (the gradient used by Vettore et al. (30)) to improve separation of platelets and reticulocytes from the mature RBCs that were the object of this study. Finally RBCs were washed (five times, RPMI 1640 medium) before preparation of membrane and cytoplasmic fractions.

RBC Purity—RBCs from five separate purifications were diluted (RPMI 1640 medium) for counting (by hematocytometer, taking the mean of five fields, repeated three times). Counts consistent with reference data for mouse RBCs were obtained (6.00–9.00 × 10⁹ cell/ml). Conventional slides of processed (five batches) and unprocessed blood, stained with Azure B (31) or May-Grünwald Giemsa (32), were counted for white blood cells (WBCs), granulocytes, monocytes, reticulocytes, and platelets.

Sample Preparation/Analysis—Preparation and analyses were essentially as described previously for human RBCs (29). Briefly membrane fractions were obtained by hypotonic phosphate buffer lyses, and soluble fractions were obtained through repeated freeze/thaw cycling. Proteins in both membrane and soluble fractions were further separated by SDS-PAGE; membrane samples were also extracted using combinations of sodium carbonate, ethanol, and EDTA. Samples were enzymatically digested either in solution or in gel (33) prior to LC-MS/MS using a PE Sciei QSTAR Pulsar quadrupole orthogonal time-of-flight mass spectrometer (QSTAR; 17 runs) or Thermo Electron hybrid two-dimensional linear quadrupole ion trap–FTICR mass spectrometer (LTQ–FT; three runs); MS/MS spectra were searched against the non-redundant International Protein Index (IPI) mouse sequence database (40,613 sequences, 17,542,708 residues; release, April 5, 2004) (34) using Mascot software (35) (version 2.0) using the same parameters as used for the human RBC proteome (29). Spectra were also searched using the corresponding reverse database to estimate false positive peptide identifications (0.5%). Because annotation databases are by nature incomplete and changing it is imperative that outputs are subjected to critical evaluation to ascribe for example the most likely function(s) when several are available. Annotation data presented here result from assembly of all information available via UniProt (Swiss-Prot and TrEMBL) (36) and Ensembl databases (European Institute for Bioinformatics (EBI) eEnsembl accessed April 1, 2007) using protein accession numbers. As such databases are incomplete and dynamic, such analyses cannot be absolute. When in doubt, a further cross-check was provided by submitting the protein description to PubMed (United States National Library of Medicine, National Institutes of Health) and evaluating the resulting literature for relevance to RBC (supplemental Table 1, column AG). Practical limits preclude citation of all relevant studies; as might be expected findings were sometimes contradictory.

Validation by MSQuant (version 1.4.1; official release, June 3, 2007), an open source software developed in our laboratory at the Centre for Experimental Bioinformatics (University of Southern Denmark), provided a manual score and spectrum evaluation for each of the peptides that had led to the identification of a given protein. The same stringent criteria were applied as for the human RBC proteome (29). Proteins were then BLAST searched (all versus all; cutoff 95% to remove redundancy). Swiss-Prot/TrEMBL (36), Ensembl, and Gene Ontology databases (37) were used for annotation. Unique Swiss-Prot/TrEMBL/Ensembl numbers provided access to sequence, isoform, family, localization, and function data for identified proteins; in parallel the protein numbers were queried against the Gene Ontology database (37), enabling grouping by class, function, or localization and quantitation within grouping (for example within the different signal transduction pathways).

An all versus all BLAST search of validated proteins was done to eliminate redundancy, providing final membrane, soluble, and mixed fraction protein lists (supplemental material). In particular, where proteins were identified only with only one method (output for each method is typically the result of three runs of pooled RBCs prepared and analyzed using the same conditions), annotation databases and the literature were extensively used to define proteins as genuine red cell components or as probable contaminants from other blood sources.

RESULTS

In material used for analyses, two WBCs, two reticulocytes, and five platelets were maximally detected per 10⁶ RBCs; granulocytes and monocytes were undetectable. Our combined purification procedures therefore reduced contaminating cell types by at least 1000-fold such that we present data for an essentially pure RBC proteome. This is confirmed by the fact that high copy number molecules CD45 (leukocyte-specific) (38), transferrin receptor (reticulocytes, lost in exosomes during maturation) (39), and ferritin receptor (40) were not in our final protein list.

The study goal was to obtain the fullest possible proteome, including low abundance proteins; these were identified using strategies we first described for the human RBC proteome (29). Due to repeat detection, highly abundant proteins such as Band 3 and spectrin (for mice 25 and 27% of the membrane proteome, respectively (41)) can provide a dynamic range challenge that reduces rare protein detection. For this and other reasons, we used parallel approaches, utilizing both the LTQ-FT and QSTAR for MS. The LTQ-FT provides rapid cycling, high mass accuracy (FT), high fragmentation speed (LF), and an additional fragmentation step (MS²) in the ion trap, whereas the QSTAR affords better quantitation statistics. For the QSTAR, exclusion lists were created. As for the human RBC proteome, LTQ-FT MS significantly enhanced the dynamic range, allowing us to identify more proteins than previously possible, including extremely low abundance extracellular RBC-binding proteins such as the complement-related factors complement C4 precursor, complement C1Q, and subcomponent subunit A.
Membrane protein identification by various extraction methods using IPI and NCBI databases

The number of proteins identified in each database is shown per extraction method. ND, not determined.

| Database          | IPI human | NCBI Mammalia | IPI mouse | NCBI Mammalia |
|-------------------|-----------|----------------|-----------|----------------|
| Not treated       | 35        | ND             | 40/93*    | ND             |
| EtOH              | 95        | 93             | 31/72*    | 30/71*         |
| Carbonate-saturated |          |                |           |                |
| Na$_2$CO$_3$ + EtOH | 48        | 38             | 54/92*    | 52/89*         |
| Na$_2$CO$_3$ × 2  | 97        | 87             | 10/27*    | 13/30*         |
| Na$_2$CO$_3$ × 2 + EtOH | 69   | 61             | 15/38*    | 18/42*         |
| Gel (combo)       | 144       | ND             | 133/278*  | ND             |

* For the mouse a distinction is made between proteins unique to the membrane fraction and proteins found in both membrane and soluble fractions.

**Comparative Analysis of Mouse and Human RBC Proteomes**

**Membrane Proteins**—40 membrane proteins (93 including proteins in the membrane/soluble fraction) were identified on initial MS analysis of digested crude RBC membranes (Table I). Because membrane proteins are often (partially) shielded from tryptic digestion by lipids and may occur as either integral or membrane-associated proteins, we assessed procedures to increase the number of detectable proteins. In contrast to human RBC membranes, ethanol solubilization/precipitation to remove lipids (42) decreased the proteins found (31 proteins; 72 including proteins in the membrane/soluble fraction). 16 proteins (47 including proteins in the membrane/soluble fraction) were found in both ETOH-treated and crude samples; G-proteins and proteasome subunits were almost exclusively present in the crude sample only.

To improve detection/differentiation between membrane-associated and integral membrane proteins, we combined ETOH treatment with sodium carbonate extraction at two concentrations (43). We compared EtOH solubilization and calcium carbonate extraction alone and combined. As for human RBC membrane samples, fewer proteins were detected using saturated carbonate procedures; loosely associated membrane proteins (e.g. Rab) disappeared with more intense carbonate extraction (mouse membrane only; supplemental material). The fewest proteins were identified using double carbonate extraction; numbers of known integral membrane proteins were stable independent of treatment suggesting that many mouse RBC membrane proteins are membrane-associated rather than integral (see Band 3, stomatin, and urea transporter in mouse membrane only; supplemental material). The abundant, tightly membrane-associated 55-kDa erythrocyte membrane protein (44) followed this trend. Consistent with our observations on human RBCs, glycosylphosphatidylinositol-anchored proteins such as CD59 glycoprotein and ADP-ribosyltransferase 4 showed constant peptide numbers across all carbonate extractions. SDS-PAGE fractionation (gel slicing) improved detection to 133 membrane proteins (278 including proteins in the membrane/soluble fraction) (Table I) and gave a molecular size window.

**Extracellular, Membrane-associated Proteins**—19 membrane proteins were probably of extracellular origin. Among these serum albumin (binds also to WBCs (45–47)) and clusterin (complement inhibition (48)) were common to human and mouse data sets. Cathepsin E (49) and B (50) were found here; Cathepsin G (51) was found on human RBCs (29). RBCs interact with plasma lipoproteins through apolipoprotein B, allowing RBC membrane remodeling (52). L-apolipoproteins are thought to be absent in rodents (53); however, we detected a widely expressed (heart, endothelial cells, and bone marrow) mouse protein similar to apolipoprotein L (Swiss-Prot accession number Q8BUC6) that is believed to affect lipid movement and/or allow lipid binding to organelles. Some low abundance proteins we identified are expressed by endothelial cells (such as phosphoribosyl-pyrophosphate synthetase-associated (54) and claudin-13 (55) and are attributed (e.g. by Swiss-Prot) to extracellular space. Although minor endothelial cell contamination cannot be excluded, RBCs express a phosphoribosyl-pyrophosphate synthetase-associated protein, suggesting that similar association may occur in RBC membrane. SPARC (secreted protein acidic and rich in cysteine) (which is secreted by megakaryocytes with a possible role in hematoipoiesis (58)) and collagen (binds to CD36 (59) and present both on erythrocytes and platelets) were also identified as was prosaposin (secreted by liver; found in various organs including nervous system (60) and may transfer gangliosides from liposomes to erythrocyte ghost membranes (61)). In contrast to human RBC binding of covalent C3b2-IgG complexes (62, 63) and serum albumin (binds also to WBCs (45–47)) and clusterin (complement inhibition (48)) were common to human and mouse data sets. Cathepsins E (49) and B (50) were found here; Cathepsin G (51) was found on human RBCs (29). RBCs interact with plasma lipoproteins through apolipoprotein B, allowing RBC membrane remodeling (52). L-apolipoproteins are thought to be absent in rodents (53); however, we detected a widely expressed (heart, endothelial cells, and bone marrow) mouse protein similar to apolipoprotein L (Swiss-Prot accession number Q8BUC6) that is believed to affect lipid movement and/or allow lipid binding to organelles. Some low abundance proteins we identified are expressed by endothelial cells (such as phosphoribosyl-pyrophosphate synthetase-associated (54) and claudin-13 (55) and are attributed (e.g. by Swiss-Prot) to extracellular space. Although minor endothelial cell contamination cannot be excluded, RBCs express a phosphoribosyl-pyrophosphate synthetase-associated protein, suggesting that similar association may occur in RBC membrane. SPARC (secreted protein acidic and rich in cysteine) (which is secreted by megakaryocytes with a possible role in hematoipoiesis (58)) and collagen (binds to CD36 (59) and present both on erythrocytes and platelets) were also identified as was prosaposin (secreted by liver; found in various organs including nervous system (60) and may transfer gangliosides from liposomes to erythrocyte ghost membranes (61)). In contrast to human RBC binding of covalent C3b2-IgG complexes (62, 63), in mice elements of the classical complement pathway were evident.

**Soluble Proteins**—Soluble proteins were analyzed three times by LTQ-FT following SDS-PAGE fractionation. To maximize low abundance hits, sensitivity was increased by filling the ion trap to capacity with one of five mass ranges in turn. Although mouse RBCs have fewer membrane proteins than human RBCs, the soluble proteome is roughly the same size.

**Detection and Validation, Including Isoforms and Protein Families**—To confirm that multiple members of the same protein family were present, unique peptides were identified wherever possible. Peptide spectra (scoring over 30) were checked for correct attribution of amino acid sequence, leading to unequivocal attribution of splice isoforms for seven of 20 membrane proteins, six of 17 soluble proteins, and four of 11 mixed fraction proteins. For example, splice isoform 2 of Q61469 lipid phosphate phosphohydroxylase 1 shared peptides NYSTNHEP and EEDPTHTLHETASSR with isoform 1 but had peptides YPYHDSTIPSRR and GFFCTDNSVK, both unique to isoform 2 (Fig. 1, A and B).
FIG. 1. Protein isoforms in RBC membranes. A, summary of identified splice isoforms with peptides that permitted unequivocal attribution to a specific isoform in bold and the further peptides identified in regular font. B, a partial ClustalW alignment for splice isoforms of lipid phosphate phosphohydrolase 1 (LPP1) proteins shown as an example of the way by which the presence of specific peptides unique to an isoform was ensured. *, identical amino acid residue; :, synonymous amino acid replacement; :, non-synonymous amino acid replacement; empty space, completely different amino acids.

| Subcellular Fraction & IPI | Splice Isoform & Protein Description | Isoform specific peptides & Unique Peptides |
|---------------------------|--------------------------------------|------------------------------------------|
| Membrane Only             |                                      |                                          |
| IPI00114558.1             | 1 of Ras-related protein Rab-1A      | SSMDPFLVYLFK                            |
|                           |                                      | EFADNLLEFPLETISAK                       |
|                           |                                      | EQWDDVADGGER                            |
| IPI00223459.1             | 2 of Lipid phosphate phosphohydrolase 1 | SYSTTSNVK                                |
|                           |                                      | EHDWTTWTSTHASSR                         |
|                           |                                      | YPHHSYTPSR                              |
|                           |                                      | CMVTDNNXK                                |
|                           |                                      | LGKVYVPQOR                               |
| IPI00308812.1             | 2 of Panoptus                    | MSAQPQNLNLK                             |
| IPI00120359.0             | 3 of Kinesin-like protein KIF1B      | OEEHALAVLYK                             |
|                           |                                      | BWNNYGINAVMDEK                         |
|                           |                                      | QWQAPNLK                                 |
| IPI00381413.1             | 3 Mitochondrial inner membrane protein | SSTEALAK                                    |
| IPI00122549.1             | PI-VDAC1 of Voltage-dependent anion channel 1 | VNGNLVKYK                                |
|                           |                                      | LTRQIFSPNYGK                            |
|                           |                                      | MSSFNYLLWQMQMVFTTYADL.GK                |
| IPI00196885.3             | Long of Protein-tyrosine phosphatase alpha precursor | VNNLPPFLSALVSK                          |
|                           |                                      | RGQLSDHSRR                              |
|                           |                                      | YDPELDW                                 |
| Mixed Fraction            |                                      |                                          |
| IPI00153407.2             | 1 of Bioconin hydrolyase            | CWHSCLNVRM                              |
|                           |                                      | BQYQVTYKVR                              |
| IPI00117710.1             | 1 of Osmotic stress protein 94      | CTPACNSLGQR                             |
|                           |                                      | MGNETCDAYDR                              |
| IPI00200444.1             | 2 of Tropomyosin alpha 3 chain      | MELQNEQK                                |
|                           |                                      | LVVEGSLR                                |
|                           |                                      | QAEPRVSNLR                               |
|                           |                                      | AGTITVAVKR                               |
|                           |                                      | TIDQLEDK                                 |
| IPI00120761.3             | Erythrocyte Band 3 anion transport protein | DHHEVLEIPOR                              |
|                           |                                      | BSEELEENIQGQAVR                         |
|                           |                                      | ANPCREPVQVTFQG                           |
|                           |                                      | SQILQWVEAANWCLGLEDNR                     |
| Soluble Only              |                                      |                                          |
| IPI00113661.0             | 1 of Proteasome activator complex subunit 3 | ITSEAEIDLYAFNFPK                       |
|                           |                                      | TTVTYEDEK                               |
|                           |                                      | NOYQYVHIHLKL                             |
| IPI00222629.1             | 1 of Sulfotransferase   Ala           | DDPFTVYKPT                              |
|                           |                                      | ELSFVEADVAYR                            |
|                           |                                      | KLYLINDRER                               |
| IPI00405001.1             | 2 of SH3-domain kinase binding protein 1 | FEGYLPAPMIPPAHGAQ                      |
|                           |                                      | TTTTFTGLYR                               |
|                           |                                      | TGDIPSTIK                                 |
| IPI00225787.5             | 3 of Mitogen-activated protein kinase 4 | THGWVAAAQVYR                             |
|                           |                                      | FYNATPRWR                                |
|                           |                                      | ESDRDLES                                 |
| IPI00126940.1             | Long of Adenosine kinase             | VPTLINSAPFQKQQFQ                        |
|                           |                                      | MAADFPK                                  |
| IPI00114783.1             | LYN A of Tyrosine-protein kinase LYN | OQRPVEPVHEPILGPR                        |
|                           |                                      | SDVQSCGLYFVYTGK                        |
|                           |                                      | DYPQEHDCGQIK                             |

B

sp|Q61469|LPP1_MOUSE  sp_vs|Q61469-2|Q61469  MFDKQFLFYVALDVCIYLAVLGLFILTA5S-RTTFQGQFPCDNSKXYPY  MFDKQFLFYVALDVCIYLAMPKITLQGLVPFFQGQGFCTDNSQNYPY  **************************************************|***** | **:****:**|**********|**********  

sp|Q61469|LPP1_MOUSE  sp_vs|Q61469-2|Q61469  RDEDNPRALLGGIVFPIC1TVMSGIESLVFSYVFNLHSLPFGHIPYLATY  RDEDNPRALLGGIVFPIC1TVMSGIESLVFSYVFNLHSLPFGHIPYLATY  **************************************************|***** | **:****:**|**********|**********  

sp|Q61469|LPP1_MOUSE  sp_vs|Q61469-2|Q61469  KAVIPEVFVSASQDIASKKHILQCSLDRPFHJAAAPSCKINSVDGYTE  KAVIPEVFVSASQDIASKKHILQCSLDRPFHJAAAPSCKINSVDGYTE  **************************************************|***** | **:****:**|**********|**********  

sp|Q61469|LPP1_MOUSE  sp_vs|Q61469-2|Q61469  DYCQGNEKEVQERGLYLFSYHSSFQSMYCHLFLAVLYLQARHGWDARLRL  DYCQGNEKEVQERGLYLFSYHSSFQSMYCHLFLAVLYLQARHGWDARLRL  **************************************************|***** | **:****:**|**********|**********  

sp|Q61469|LPP1_MOUSE  sp_vs|Q61469-2|Q61469  PMLQPPGIAFSYVVGL6RSDFIKHNSDVTQGL1QGAANAILVALYVSDF  PMLQPPGIAFSYVVGL6RSDFIKHNSDVTQGL1QGAANAILVALYVSDF  **************************************************|***** | **:****:**|**********|**********  

sp|Q61469|LPP1_MOUSE  sp_vs|Q61469-2|Q61469  FKKDSYKEREDEPRLHTETASQHSTSEER  FKKDSYKEREDEPRLHTETASQHSTSEER  **************************************************|***** | **:****:**|**********|**********
For cytoskeleton components, isoforms/members of the main protein families were similar in human and mouse proteomes. Thus the same ankyrin and spectrin isoforms were unequivocally identified in both RBC proteomes (Table II). Similar profiles emerged for tropomyosin and actin, although tropomyosin 2 was unique to mouse RBC. Tropomyosins interact in an isoform/member-dependent manner with tropomodulin; defects in expression can result in RBC shape abnormalities (64). Actin cytoplasmic 2 (γ-actin) appears to be a mouse, but not human, mature RBC protein. Increased γ-actin in human RBCs (induced by high circulating glucose levels) may pathologically reduce RBC deformability (65). Glucose effects on mouse RBC may consequently differ from human RBC. Adducins α and β occur in both proteomes; adducin γ was detected only in mouse RBC. β-Adducin knock-out mice show compensatory overexpression of γ-adducin, leading to spherocytic hereditary elliptocytosis (66). In the analysis of the human RBC tubulin family members only α-3 could be identified with certainty; this is missing in the mouse RBC proteome. Due to the considerable identity in amino acid stretches between tubulin β-5/β-1 and α-6/α-1, peptides from these RBC proteins could not be attributed unequivocally to one or the other tubulin family member in humans, whereas attribution was possible in the mouse where tubulin β-5 and α-1 are definitely present. Interestingly ExPASy (36), Ensembl, and PRIDE (Proteomics Identifications database) (67) only contain a "similar to tubulin β-1" sequence that differs significantly from mouse tubulin β-5 (Table II).

There is debate as to whether human erythrocytes possess actin capping protein. In accord with published reports (68, 69), we detected F-actin capping protein in both human (α and β subunits; unique to membrane fraction) and mouse (α subunit only; membrane and soluble fractions) RBCs. Thus, combined evidence suggests that such a protein is present in RBCs, although uncertainty concerning state of activity/role remains (70, 71).

**Human/Mouse Comparison: Orthologs and Family-related Proteins**—Having eliminated redundancy, final human and mouse RBC protein lists were compared with identify orthologs and family-related proteins. The mouse proteome comprising uniquely membrane (MM; 341 proteins) and uniquely soluble (HS; 252 proteins) sets was compared with the human RBC proteome comprising uniquely membrane (HM; 341 proteins) and uniquely soluble (HS; 252 proteins) protein sets (Fig. 2). Determination of commonality/ uniqueness and evolutionary relationships used a BLAST homology algorithm based on National Center for Biotechnology Information (NCBI) BLAST.exe and algorithms by Bork and coworkers (72–74). As no algorithm reliably defines orthologs, we used orthology/paralogy predictions generated by the European Bioinformatic Institute through a pipeline where maximum likelihood phylogenetic gene trees (generated by an algorithm to estimate large PHYlogenies by Maximum Likelihood (PHYML)) play a central role (75–77). This reconciles gene trees with species trees (using RAP), annotating internal nodes distinguishing duplication/speciation events, to repre-
sent an evolutionary history for gene families. Although there is a clear concordance with reciprocal best approaches in the simple case of unique orthologous genes, this approach also finds more complex one-to-many and many-to-many relationships. In this study, proteins were defined as orthologs when homology BLAST returned >80% identity and the algorithm tree of Bork and co-workers (72–74) showed a common precursor or when EBI predictions indicated that two proteins were orthologs. These combined approaches returned orthologs as follows: 56 mouse soluble/HS, 21 mouse soluble/HM, 110 MM/HM, 11 MM/HS, 53 MM&S/HM, and 60 MM&S/HS. 187 mouse soluble, 88 MM, and 39 MM&S proteins were unique. These and family-related proteins (nine mouse soluble/HS, one mouse soluble/HM, four MM/HS, and 32 MM/HM) are detailed in the supplemental material.

Annotation

To render human and mouse data sets as comparable as possible the final protein lists were evaluated in depth using all available literature and databases. Because single proteins may have multiple functions/locations, little or no information may be available for a given protein, and databases are dynamic and may not recognize submitted queries (different accession numbers or protein descriptions), all annotations were manually reviewed using consistent criteria, ensuring maximal comparability between human and mouse proteomes. A Gene Ontology (37) annotation of some sort was found for 535 of 668 proteins detected; proteins were classified as involved in cellular (188 proteins), physiological (183 proteins), or regulatory (34 proteins) processes. 130 were unclassified.

Membrane Components—To enable comparison, all MM proteins and HM orthologs in the MM&S fraction were treated as a single set (subcellular localization is shown in Fig. 3). For six proteins with double annotation both localizations were attributed. Visual inspection of equally loaded SDS-PAGE gels suggested the mouse membrane RBC proteome to be less complex than the human equivalent (Fig. 4); our annotation confirms this. Extending the analysis to include MM&S-unique and mouse soluble proteins with HM orthologs does not alter the finding. In fact, most proteins in these two sets are cytoplasmic, cytoskeletal, or organellar; only five of 61 such proteins are classified as integral membrane proteins.

Mouse and human RBC membranes appear to differ substantially in the number of integral membrane proteins and the degree and tightness of association of cytosolic proteins (e.g. glycolytic enzymes and carbonic anhydrase II) to the membrane network. Fewer proteins involved in glycolysis were associated with mouse membranes (three proteins) in comparison with human membrane (eight proteins) (Table III). As for human RBC, with the exception of glyceraldehyde dehydrogenase, cytoskeleton removal protocols not only resulted in fewer peptides from cytoskeletal and cytoskeleton-asso-
ciated proteins but also reduced hits for almost all glycolytic proteins. This is in accord with data from various mouse strains showing that inward facing membrane-associated enzymes function by carrying the organization of the membrane into the cell interior and that few glycolytic enzymes known to stably bind to the human RBC membrane do so in mice (78, 41).

As for the human RBC proteome, most membrane proteins are involved in binding (156 proteins) and have catalytic activity (112 proteins). Many show transporter (67 proteins), signal transducer (37 proteins), or structural activity (22 proteins). Transport (Fig. 5A) and metabolic (Fig. 5B) activities are diverse. As for the human proteome, probable reticulocyte legacies such as intracellular transport proteins of the Golgi, endoplasmic reticulum, and mitochondrion were detected. This supports the concept of a scheduled RBC protein degradation process for RBC maturation and aging that is conserved among mammals. An ortholog for vesicle trafficking protein SEC22b identified in the human proteome was absent in mouse RBC. However, vesicle-associated membrane protein 5 present in the mouse-unique set is a protein with very similar characteristics: it belongs to the synaptobrevin family and has the same trafficking function (79–81). Thus although direct human-mouse orthologs are not always present, functionally equivalent proteins of different origin may perform similar tasks in RBCs of the two species.

Although RBCs are devoid of nuclei, three proteins annotated to have transcription regulator activity (two proteins) and translation regulator activity (one protein) were identified. Closely similar sets of organelar proteins occurred in both proteomes. Some migrated at anomalously high molecular weight (elongation factor A protein 1 and eukaryotic translation initiation factor 2C 2) and may be inactive (see below). Some may have roles in mature RBCs other than so far ascribed. Ribosomal proteins S19 (MM) and S27 (HM) and 40S ribosomal proteins S3 (mouse soluble) and S6 (HS) may provide one example. rpS3 and rpS6 interact with heat shock protein 90 (Hsp-90) thereby preventing ubiquitination and proteasome-dependent degradation (82). A further regulatory mechanism involves Hsp-70, which associates with free rpS3 promoting its degradation (83). Both chaperones occur in mature red blood cells (83) and were found in this study.

We analyzed regulation of cellular processes, physiological processes, and enzyme activities. For cellular processes, as for human RBC, the most common regulatory activity involves programmed cell death, presumably a legacy of prior development (84), followed by involvement in regulation of transport and signal transduction. 12 proteins are involved in general signal transduction, seven are linked to receptor signaling pathways, and 24 have a role in the intracellular signaling cascade. Three proteins are ascribed by databases as being part of a phosphorelay (two-component signal transduction system). Of proteins involved in surface receptor-linked signal transduction, five belong to the G-protein-coupled receptor protein signaling pathway, two belong to the acetylcholine receptor signaling muscarinic pathway, and one belongs to the integrin-mediated signaling pathway; the intracellular signaling cascade is itself divided into protein kinases (four pro-
teins), second messengers (one protein), and small GTPases (19 proteins). Such a high representation of signal transduction proteins seems unlikely to be purely a reticulocyte legacy and is in line with data for the human RBC (29). The predominance of small GTPases agrees with data showing that the two α subunits of Gα and Gβ and the Rab proteins function together with an unknown G-protein (85); our data suggest this may be the α inhibiting activity polypeptide 2. Further to cellular processes, we identified proteins regulating cell shape (ezrin, moesin, and phospholipid scramblase 3), cell adhesion (two proteins), complement activation (two proteins), cell volume (one protein), and cell redox homeostasis (one protein). Amyloid β A4 protein, which appears to be involved in a great variety of processes, was present in both mouse and human RBCs.

Diverse mechanisms/proteins including vascular processes, regulating blood vessel size, and coagulation fall under physiological regulatory processes. Protein sets involved in these processes are very similar to those identified in the human RBC proteome, including those involved in nitric oxide

| Glycolysis enzymes | MOUSE | HUMAN |
|--------------------|-------|-------|
|                    | Protein Description | Protein Description |
|                    | Membrane fraction   | Membrane fraction   |
| ortholog_one2one   | IPI00122684.1 enolase 1, alpha non-neuronal, glycolysis | IPI00215736 Alpha enolase |
|                    | ortholog_one2one    | IPI00221402.3 fructose-bisphosphate aldolase C homolog (aldolase 1, A isoform) | IPI00418262 ALDOC protein |
|                    | ortholog_one2one    | IPI00125439.3 similar to glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (phosphorylating) - muscle | IPI00383758 Glyceraldehyde-3-phosphate dehydrogenase, muscle |
|                    |                    | Mixed fraction, but prevalent in soluble |
| apparent ortholog_one2one | IPI00407130.1 Pyruvate kinase, M2 isozyme | IPI00220644 Splice isoform M1 or M2 of P14618 Pyruvate kinase |
| ortholog_one2one   | IPI00230002.3 phosphoglycerate kinase | IPI00169383 Phosphoglycerate kinase 1 |
|                    | Soluble Fraction    |
|                    | ortholog_one2one    | IPI00221402.6 Fructose-bisphosphate aldolase A |
|                    | ortholog_one2one    | IPI00319731.1 Glyceraldehyde-3-phosphate dehydrogenase |
|                    | ortholog_one2one    | IPI00331541.3 Phosphofructokinase, muscle |

**Table III**

Glycolytic enzymes in the membrane fraction of human and mouse RBCs
modulation (86, 87). These comprise inhibitory G-proteins (guanine nucleotide-binding protein G, and inhibiting activity polypeptide 2), which block nitric oxide-mediated ATP release from RBCs (88) (and have a further role in regulation of the AQP1 water channel (89)) and Hsp-90, which modulates nitric oxide biosynthesis. RBC nitric-oxide synthase (NOS) has been reported to be membrane-bound (P-face) (90, 91), but reports disagree on the nature of the NOS. Although Kleinbongard et al. (90) described it as a 140-kDa endothelial NOS (eNOS) Bhattacharya et al. (91) described it as an insulin-activated NOS comprising heavy (135 kDa) and light chains (95 kDa). Analysis of our data for evidence of NOS expression showed low confidence-scoring peptides mapping to an inducible NOS (human, FDVVPLVLQANGR; mouse, VVF-FASMLMR) in the soluble mouse and human RBC proteomes. Initially excluded as low level contaminants, it may be that RBC eNOS diverges from eNOS as annotated in the databases and shows partial similarity to inducible NOS and that this 135-kDa protein is the heavy subunit of the insulin-activated NOS reported by Bhattacharya et al. (91). By analogy with protein-tyrosine phosphatase receptor type C-associated protein (PTPRC) shown to have a role in the insulin-mediated activation of NOS in monocytes (92) and endothelial cells (93), the low abundance RBC membrane-based receptor-type tyrosine-protein phosphatase we identified in both proteomes would be the insulin-reactive 95-kDa light chain.

**Soluble Components**—To enable comparison, all mouse soluble proteins and HS orthologs in the MM&S fraction were treated as a single set. Of 292 unique soluble proteins identified, 228 were unequivocally annotated as cytoplasmic, 10 were unequivocally annotated as cytoplasmic and nuclear, eight were unequivocally annotated as Golgi/ER and/or endoplasmic reticulum, and seven were unequivocally annotated as mitochondrial and/or cytoplasmic (Fig. 6). Those annotated as both cytoplasmic and organelar appeared to have trans
porter roles, accounting for the distribution. Although different
numbers of total soluble proteins were identified for mouse
and human RBCs (292 and 252, respectively) more proteins
appear to be metabolized in the mouse (47 and 20, respecti-
vely) making the difference between the two proteomes (245
and 232, respectively) much less significant.

As for the human proteome, most soluble proteins were
implicated in cellular metabolism and/or transport; prevalent
functions were proteolysis (17 proteins), ATPase (eight pro-
teins), and dehydrogenase (34 proteins) activity. Most
proteins involved in metabolic processes were catabolic (27
proteins); this is not surprising given the RBC life history and
is concordant with what was seen for human RBC. A further
17 proteins were involved in household metabolism (GSH,
nucleotide (ATP and ADP), and glucose) and in macromole-
cule metabolism, whereas only a few proteins were involved in
cellular biosynthesis (five proteins). The complex with most
members was the proteasome (17 proteins). RBC household
complexes (ubiquitin ligase complex, 6-fructokinase complex,
Hb complex, and phosphopyruvate hydratase complex) were
also present along with complexes probably resulting from
incomplete degradation of molecules no longer functionally
required (RNA complex, DNA factor A complex, nucleosome,
and ribonucleoprotein complex). Although household com-
plexes migrated at the expected molecular weight, residual
complexes did not (as defined by UniProt (36)). Some residual
complexes (e.g. nucleosome and ribonucleoprotein complex)
were detected in both mouse and human RBCs, suggesting
that common catabolic pathways and schedules for RBC
maturation exist.

Not All Proteins Are Present in Their Active Form—Although
our preparation methods focused on purification of mature
RBCs, the protein content is likely to vary with cell age; RBCs
may be considered as "in development" because degradation
of organelles occurs during maturation, and chemical and
enzymatic modifications occur during cellular aging. "Resid-
ual" proteins may therefore be detected, and it becomes
important to know their state. This thesis, first presented in
analysis of our human RBC proteome, is supported by the
mouse RBC proteome data. As before, we compared ex-
pected and observed molecular weights for proteins in ques-
tion to obtain information on their status (Fig. 7). Of 192
proteins identified after in-gel separation and digestion, 16
migrated faster than expected, and 38 migrated slower than
expected in a gel fraction also containing ubiquitin. High
apparent molecular weights may reflect ubiquitination (known
for spectrin (94)) and/or oligomerization in aging RBCs (95).
Several proteins migrated anomalously in both MM and HM
(78-kDa glucose-regulated protein precursor, elongation fac-
tor 1-α 1, importin 9, 14-3-3 protein family, transforming pro-
tein p21, ATP-binding cassette subfamily B, member 6, and
Hsp-90). We hypothesized (29) that the molecular weight shift
may be attributable to complexes resulting from reduction-
insensitive protein-protein interactions. Although the molecu-
lar weight shift is often similar, indicating a common degra-
dation plan (strategy and timing), exceptions occur, allowing
us to exclude coincidental behavior due to the in-gel migra-
tion properties of the different proteins. Thus, the 78-kDa
glucose-regulated protein precursor is degraded in mice
(lower molecular weight) and probably ubiquitinated in hu-
mans (higher molecular weight); both 14-3-3 proteins migrate
at high apparent molecular weight with ubiquitin in mice; in
human RBCs one protein is probably ubiquitinated, and the
other is degraded. Control proteins occurred in fractions cor-
responding to the expected molecular weight, whereas Band
3, spectrins, and other cytoskeletal proteins occurred at sev-
eral molecular weights as reported previously (96). Of soluble
proteins also analyzed for in-gel migration, 35 were unique to
the mouse RBC proteome; 23 migrated slower than expected
in fractions also containing ubiquitin, 19 migrated faster than
expected, and 197 were found at the expected molecular
weight (soluble protein not at molecular weight/soluble pro-
teins at molecular weight; supplemental material).

Although mature RBCs are devoid of both nucleus and
internal organelles, organelar proteins common to both hu-
mans and mouse RBCs were identified usually in the same
metabolic state. Golgi proteins (nicastrin, adaptor protein
complex, and copper-transporting ATPase), ribosomal pro-
teins (40 S ribosomal proteins S6 and S3), and some endo-
plasmic reticulum proteins (EPR29, reticulin 3, and protein
-disulfide isomerase A3) migrate at expected molecular
weights. In contrast, most nuclear (nucleosome assembly
proteins 1-like 1 and 1-like 4 and elongation factor 1) and
mitochondrial proteins (ATP-binding cassette subunit B,
member 6; ATP synthase β chain; malate dehydrogenase;
and protein NipSnap 2) appear to be degraded or ubiquiti-
nated/polyubiquitinated. However Ras-related proteins ap-
peared predominantly degraded in the human RBC but at the
expected molecular weight in the mouse, and calreticulin

![Mouse membrane gel with molecular weight indicator.](image)

Shown is a typical mouse (Mr) membrane SDS-PAGE gel with
the relative size makers (M) giving an example of a few proteins that
presented an apparent molecular weight different from the expected
molecular weight.
appears to be polyubiquitinated in human RBC but at the expected molecular weight in mouse RBCs (supplemental material).

**DISCUSSION**

Advanced LC-MS technology, coupled with biochemical procedures for sample preparation and new bioinformatics tools, has been used to derive a normal mouse RBC proteome 72–96 h after blood collection. Using stringent purification procedures to isolate RBCs and detection limits of about 500 copies of protein/cell we present the most complete analysis of the mouse RBC proteome undertaken to date. To provide for comparability with our recently published in-depth analysis of the human RBC proteome (29), we ensured that the harvesting of the RBCs, their downstream processing (biochemical procedures), the type of MS analysis (MS machines, time, and HPLC gradients), and the validation parameters were fully equivalent between the two data sets. Thus, for example, we decided to maintain use of the Percoll-based reticulocyte depletion protocol used by Vettore et al. (30) in both studies rather than switch to implement a novel Percoll-based method (97) midway between the studies. Although this novel method is reported to be superior to fluorescence-activated cell sorting and magnetic cell sorting depletion, it was not compared with existing Percoll-based methodologies including that of Vettore et al. (30), and there are to date no reports of its use in proteomics study. Using this conservative approach has allowed an in-depth comparison of human and mouse RBC components, a comparison only possible when components of both proteomes can be unequivocally identified. A recent minireview (98) provides an overview of all human RBC proteins found to date by different authors. Unfortunately for the purposes of comparison between our findings and those reported in this review, this review did not incorporate accession numbers nor were the proteomics methodologies leading to protein identification described in sufficient detail to make comparison with our list feasible.

Using these conservative approaches and with the caveat that, due to the nature of the study, inclusion of a small number of false positive identifications or incorrect assignment of contaminating proteins cannot be precluded, after validation 247 solely membrane and 232 solely soluble proteins were identified; 167 proteins were in both membrane and soluble fractions. Proteins were scanned for likely physiological role(s) in RBC, for subcellular localization, and molecular function. Isoforms were critically screened for unique peptides, and all versus all BLAST removed redundancy from final lists. Proteins were subsequently assessed for probable metabolic status by comparing expected with apparent molecular weight. Anomalous migration may be expected in reticulocyte legacy proteins; migration faster than expected was taken to indicate degradation, whereas co-migration with ubiquitins at elevated molecular weight was suggestive of non-functional protein incompletely proteolyzed by proteasomes. At 60 days, the life span of mouse RBC is half that of human RBC (99), and mouse blood has a higher reticulocyte content than human blood. Taken together these observations, which are concordant with the high red blood cell turnover rate (100) and higher overall metabolism in the mouse, explain the higher content of metabolized residual proteins (at different molecular weight than expected) found in the mouse soluble RBC fraction. The higher reticulocyte content of mouse blood leads to a relatively younger population of mature RBCs in mice (101, 102) than in humans. Such RBCs are likely to contain more reticulocyte legacy proteins that have not (yet) been fully degraded and, as shown here, are therefore revealed by MS.

Using algorithms to identify orthologs and family-related and unique proteins, a comparison of human and mouse RBC proteomes revealed many orthologs likely to share function. Where direct human-mouse orthologs were missing, proteins could be identified for which related functions have been reported. These may function similarly in the two RBC species.

Strikingly protein isoforms of the cytoskeletal network were frequently common to human and mouse; differences were consistent with prior literature. Although RBCs lack organelles, the same organelle proteins occur in human and mouse RBCs. Apart from rare exceptions these are in the same metabolic state, suggesting that some organelar proteins may have (short lived) relevance even beyond the reticulocyte stage and share a common plan/schedule for metabolic degradation. Our findings underpin the hypothesis (29) that maturation of RBCs from reticulocytes and aging of RBCs are ongoing processes that continue their whole life span. The RBC thus appears to be a dynamic blood component, and with these data further biochemical studies can be directed to unlocking further surprises in RBC behavior. The number of membrane-associated proteins described in this study suggests that this may be especially true in the context of their interplay with other cells and plasma proteins (103–106). A detailed summary of all proteins, their metabolic form, their isoforms, and most relevant peptides found and an ortholog table is available as supplemental material; a database is also being created.

**Acknowledgment**—We acknowledge the help of Dr. Abel Ureta-Vidal EnsEMBL Compara Project Leader, European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute.

* This work was supported by Netherlands Organization for Scientific Research (NWO Genomics Grant 050-10-053), by the Danish National Research Foundation, and by BioMalPar Contract CT 2004-503578. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

$\text{The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.}$

|| To whom correspondence should be addressed. Tel.: 31-15-284-2538, Fax: 31-15-284-2600; E-mail: thomas@bprc.nl.
REFERENCES

1. Mouse Genome Sequencing Consortium (2002) Initial sequencing and comparative analysis of the mouse genome. Nature 409, 520–562.
2. Yazdanbakhsh, K., and Scaradavou, A. (2004) CR1-based inhibitors for prevention of complement-mediated immune hemolysis. Drug News Perspect. 17, 314–320.
3. De Franceschi, L., Rivera, A., Fleming, M. D., Honczarenko, M., Peters, L. L., Gascard, P., Mohandas, N., and Brugnara, C. (2005) Evidence for a protective role of the Gardos channel against hemolysis in murine hereditary spherocytosis. Blood 106, 1454–1459.
4. Fabiani, G., Vargues, R., and Fulchiron, G. (1952) Results of the intracardiac or intraperitoneal injection in white mouse of large quantities of erythrocytes parasitized with Plasmodium berghei. C. R. Seances Soc. Biol. Fil. 146, 437–440.
5. Reagan, R. L., Stewart, M. T., and Brueckner, A. L. (1982) Erythropoiesis, and phospholipid scramblase-mediated phosphatidylserine exposure. Exp. Hematol. 10, 394–402.
6. Franke-Fayard, B., Janse, C. J., Cunha-Rodrigues, M., Ramesar, J., Buscher, P., Que, I., Lowik, C., Voshol, P. J., den Boer, M. A., van Duinen, S. G., and Marien, M., Mota, M. M., Wets, A. P. M., and Waters, A. P. M. (2005) Malaria parasite sequestration: CD36 is the major receptor, but cerebral pathology is unrelated to sequestration. Proc. Natl. Acad. Sci. U. S. A. 102, 11468–11473.
7. Kean, L. S., Brown, L. E., Nichols, J. W., Mohandas, N., Archer, D. R., and Kuypers, F. A. (2000) Murine recessive hereditary spherocytosis, sph/sph, and four alternative degrees of an inherited macrocytic anemia in the mouse. Leuk. Lymphoma 37, 125–135.
8. Peters, W. (1967) Chemotherapy of Plasmodium chabaudi infection in albino mice. Ann. Trop. Med. Parasitol. 61, 52–56.
9. Min-Oo, G., and Gros, P. (2005) Erythrocyte variants and the nature of their malaria protective effect. Cell. Microbiol. 7, 753–756.
10. De Jong, K., Emerson, R. K., Butler, J., Bastacky, J., Mohandas, N., and Brookoff, D., Maggio-Price, L., Bernstein, S., and Weiss, L. (1982) Erythroid differentiation of haemopoietic stem cells. Nature 295, 257–260.
11. Yazdanbakhsh, K., and Scaradavou, A. (2004) CR1-based inhibitors for prevention of complement-mediated immune hemolysis. Drug News Perspect. 17, 314–320.
12. Sorensen, S., Rubin, E., Polster, H., Mohandas, N., and Schrier, S. (1990) Evidence for a protective role of the Gardos channel against hemolysis in murine hereditary spherocytosis. Blood 106, 1454–1459.
13. De Jong, K., Emerson, R. K., Butler, J., Bastacky, J., Mohandas, N., and Brookoff, D., Maggio-Price, L., Bernstein, S., and Weiss, L. (1982) Erythroid differentiation of haemopoietic stem cells. Nature 295, 257–260.
14. Dunn, C. D. (1971) The differentiation of haemopoietic stem cells. Nature 230, 270–276.
15. Matsuzaki, H., Bohlen, P., and Marzluff, W. F. (1994) Mechanisms of immunological tolerance loss versus erythrocyte self-antigens and autoimmune hemolytic anemia. Autoimmunity 21, 221–224.
16. Torbiani, T., and Torbiani, T. (2003) Measuring the specific activity of the CD45 protein tyrosine phosphatase. J. Immunol. Methods 277, 127–134.
17. Russell, E. S., and Fonda, E. L. (1951) Quantitative analysis of the normal and four alternative degrees of an inherited macrocytic anemia in the house mouse. J. Number and size of erythrocytes. Blood 6, 892–905.
18. Thunell, S. (1965) Determination of incorporation of 35SFe in hemoglobin of peripheral red blood cells and of red blood cells in bone marrow cultures. Clin. Chim. Acta 11, 321–323.
19. Marks, P. A., and Koch, J. S. (1966) Development of mammalian erythrocyte cells. Curr. Top. Dev. Biol. 1, 213–252.
20. Dunn, C. D. (1971) The differentiation of haemopoietic stem cells. Ser. Haematol. 4, 1–71.
21. Whitsett, C., F. (1995) The role of hematopoietic growth factors in trans-fusion medicine. Hematol. Oncol. Clin. N. Am. 9, 23–26.
22. Marks, P. A., Rifkind, R. A., and Bank, A. (1974) Control of gene expression during erythroid cell differentiation. Adv. Exp. Med. Biol. 44, 221–224.
23. Broff, D., Maggio-Price, L., Bernstein, S., and Weiss, L. (1982) Erythropoiesis in ha/ha and sph/sph mice, mutants which produce spectrin deficiency erythrocytes. Blood 59, 646–651.
24. Cole, R. J., Garlick, J., and Tarbutt, R. G. (1974) Disturbed haem and globin synthesis in reticulocytes of prenatal flexed-tailed (f-f) anaemic mice. Genet. Res. 23, 125–135.
25. Oldenborg, P. A. (2004) Role of CD47 in erythroid cells and in autoimmunity. Leuk. Lymphoma 45, 1319–1327.
26. Flogoli, E., and Torbiani, T. (2003) Mechanisms of immunological tolerance loss versus erythrocyte self-antigens and autoimmune hemolytic anemia. Autoimmunity 36, 199–204.
27. Wilson, W. E., and Talmage, D. W. (1965) Erythrocyte chimerism and acquired immunologic tolerance. J. Immunol. 94, 150–156.
90. Kleinbongard, P., Schulz, R., Rassaf, T., Lauer, T., Dejam, A., Jax, T., Kumara, I., Gharini, P., Kabanova, S., Ozyumran, B., Schnurch, H. G., Godecke, A., Weber, A. A., Robenek, M., Robenek, H., Bloch, W., Rosen, P., and Kelm, M. (2006) Red blood cells express a functional endothelial nitric oxide synthase. Blood 107, 2943–2951

91. Bhattacharya, S., Chakraborty, P. S., Basu, R. S., Kahn, N. N., and Sinha, A. K. (2001) Purification and properties of insulin-activated nitric oxide synthase from human erythrocyte membranes. Arch. Physiol. Biochem. 109, 441–449

92. Schmeisser, A., Garlichs, C. D., Zhang, H., Eskafi, S., Graffy, C., Ludwig, J., Strasser, R. H., and Daniel, W. G. (2001) Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. Cardiovasc. Res. 49, 671–680

93. Lu, X., Dunn, J., Dickinson, A. M., Gillespie, J. I., and Baudouin, S. V. (2004) Smooth muscle α-actin expression in endothelial cells derived from CD34+ human cord blood cells. Stem Cells Dev. 13, 521–527

94. Corsi, D., Paiardini, M., Crinelli, R., Bucchini, A., and Magnani, M. (1999) Alteration of α-spectrin ubiquitination due to age-dependent changes in the erythrocyte membrane. Eur. J. Biochem. 261, 775–783

95. Lutz, H. U. (2004) Innate immune and non-immune mediators of erythrocyte clearance. Cell. Mol. Biol. 50, 107–116

96. Lin, C., Cotton, F., Boutique, C., Dhermy, D., Vertongen, F., and Gulbis, B. (2000) Capillary gel electrophoresis: separation of major erythrocyte membrane proteins. J. Chromatogr. B Biomed. Sci. Appl. 742, 411–419

97. Goodman, S. R., Hughes, K. M., Kakhniashvili, D. G., and Neelam, S. (2007) The isolation of reticulocyte-free human red blood cells. Exp. Biol. Med. (Maywood) 232, 1470–1476

98. Goodman, S. R., Kurdia, A., Ammann, L., Kakhniashvili, D., and Daescu, O. (2007) The human red blood cell proteome and interactome. Exp. Biol. Med. (Maywood) 232, 1391–1408

99. Walker, W. S., Singer, J. A., Morrison, M., and Jackson, C. W. (1984) Preferential phagocytosis of in vivo aged murine red blood cells by a macrophage-like cell line. Br. J. Haematol. 58, 259–266

100. Rodnan, G. P., Ebaugh, F. G., Jr., Spivey, F. M. R., and Chambers, D. M. (1957) The life span of the red blood cell and the red blood cell volume in the chicken, pigeon and duck as estimated by the use of Na2Cr51O4. With observations on red cell turnover rate in the mammal, bird and reptile. Blood 12, 355–366

101. Gronowicz, G., Swift, H., and Steck, T. L. (1984) Maturation of the reticulocytes in vitro. J. Cell Sci. 71, 177–197

102. Rabesandratana, H., Toutant, J. P., Reggio, H., and Vidal, M. (1998) Decay-accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) are released within exosomes during in vitro maturation of reticulocytes. Blood 91, 2573–2578

103. Kooyman, D. L., Byrne, G. W., McClellan, S., Nielsen, D., Tone, M., Waldmann, H., Coffman, T. M., McCurry, K. R., Platt, J. L., and Logan, J. S. (1995) In vivo transfer of GPI-linked complement restriction factors from erythrocytes to the endothelium. Science 269, 89–92

104. Shichishima, T., Terasawa, T., Hashimoto, C., Ohto, H., Takahashi, M., Shibata, A., and Maruyama, Y. (1993) Discordant and heterogeneous expression of GPI-anchored membrane proteins on leukemic cells in a patient with paroxysmal nocturnal hemoglobinuria. Blood 81, 1855–1856

105. Civenni, G., Test, S. T., Brodbeck, U., and Butikofer, P. (1998) In vitro incorporation of GPI-anchored proteins into human erythrocytes and their fate in the membrane. Blood 91, 1784–1792

106. Sloand, E. M., Maciejewski, J. P., Dunn, D., Moss, J., Brewer, B., Kirby, M., and Young, N. S. (1998) Correction of the PNH defect by GPI-anchored protein transfer. Blood 92, 4439–4445