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HEMOGLOBIN - A NOVEL LIGAND OF HEPATOCYTE ECTOPIC F1-ATPase

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The liver is largely responsible for free hemoglobin uptake, but the molecular mechanism of this phenomenon has never been revealed. This paper presents the results of the study on hemoglobin binding components of the hepatocyte membrane that were purified using affinity chromatography on a hemoglobin matrix and identified by peptide mass fingerprinting. Both F1-ATPase alpha and beta subunits were retrieved. The binding was confirmed *via* an intrinsic fluorescence quenching study using a purified recombinant F1-ATPase beta subunit, and the dissociation constant for the complex was estimated from the saturation binding curve ($Kd = 7.5 \times 10^{-7}$ M). The results indicate that hemoglobin binds to hepatocyte ectopic F1-ATPase. We suggested the plausible role of the receptor in endocytosis of hemoglobin by the hepatocyte.

K e y   w o r d s : *hemoglobin, ectopic F1-ATPase, F0F1 ATP synthase, endocytosis, protein uptake, hepatocyte, hemoglobin receptor*

INTRODUCTION

Hemoglobin (Hb) is one of the most abundant proteins in the human body and the major component of erythrocytes, supporting their basic function as oxygen and carbon dioxide transporters. The erythrocyte life span is about 120 days. Thereafter, the senescent cells are removed from circulation by mononuclear phagocyte cells. However, the process is not complete and free hemoglobin is released into the circulation to a certain extent. After dissociation, dimeric Hb is complexed with plasma glycoprotein haptoglobin (Hp), while the undissociated tetrameric form remains in the circulation. Uptake of free Hb and Hp-Hb largely occurs in the liver (1, 2). Any excess of free hemoglobin is also rapidly removed by the kidney after glomerular filtration (3, 4).

Research into the molecular mechanism of hepatic clearance of these compounds has been in progress since the early 60's, and for decades has provided ambiguous or even contradictory results. One controversy was the cell type responsible for these processes. In some studies, uptake has been observed exclusively either in hepatocytes or in Kupffer cells, while in others it has been noted in both types of cells. There are also discrepancies concerning whether free Hb can be endocytosed by liver cells, or only when complexed with Hp.

Using histochemistry, Goldfischer *et al*. (5) demonstrated the presence of Hb in lysosomes of both hepatocytes and Kupffer cells after intravenous administration to rats. Hemoglobin uptake by hepatocytes has also been observed in studies using ^{3}H-Hb and autoradiography in mice (6). Kino *et al*. (7) found an accumulation of intravenously administered ^{59}Fe-Hb-Hp in rat liver and binding of ^{125}I-Hb-Hp complex to the isolated membranes of hepatocytes. Subsequent studies by these authors have confirmed the uptake of Hp-Hb complex in hepatocytes via immunohistochemistry; however, the studies have failed to demonstrate internalization of the Hp-Hb complex by isolated hepatocytes. The authors have suggested that the receptor for the Hp-Hb complex is sensitive to proteolysis and undergoes inactivation during isolation of hepatocytes using collagenase (8, 9). In our studies (10), hepatocytes have been isolated *via* liver perfusion with EDTA. The cells were able to both bind and internalize fluorescently labeled Hp-Hb. The determined dissociation constant for the binding reaction was 1.2 μM, and the binding was inhibited by anti-Hb antibodies. The internalization of the complex was dependent on the presence of ATP and its rate was accelerated by increasing GTP concentrations. In further studies, we have also found that isolated hepatocytes bind not only the Hp-Hb complex but also free Hb. Both labeled Hb and Hb-Hp were displaced by unlabeled Hb, suggesting that at least part of the structure recognized by the receptor is located in the Hb molecule. The binding protein was partially purified from the solubilized hepatocytes using affinity chromatography on a bed with conjugated hemoglobin. Due to the preparation on a small scale, we were not able to identify and characterize the protein (11).

Just recently that research was able to shed some light on this issue. It was demonstrated that CD163, a protein belonging to the superfamily of cysteine-rich scavenger receptors (SRCR-SF), is the functional receptor for the Hb-Hp complex. The protein had been discovered much earlier in the course of research on macrophage differentiation antigens, but its basic

function had remained unknown for a long time (12). The role of CD163 in the catabolism of hemoglobin was later confirmed by Schaer *et al.* (13), who found that not only the Hp-Hb complex but also free Hb is bound by the receptor. It should be stressed, however, that the expression of this receptor is limited only to the mononuclear-phagocytic cells, which in the liver are represented exclusively by Kupffer cells. Since these cells represent only a very small fraction of liver cells, the role of CD163 in the gross clearance of hemoglobin is open to question. Thus, it is believed that the physiological roles of these interactions are associated with receptor-mediated immunomodulation during late-stage acute or chronic inflammation (14, 15). Recent studies have also demonstrated that there are major differences in CD163-dependent scavenging systems between species, which may explain discrepancies observed by researchers in the past (16).

Our study, carried out at a later stage (17) in mice, reaffirmed Hb uptake by hepatocytes. Furthermore, internalization of the protein occurred in both wild-type and Hp knockout mice, in which the Hp pathway is obviously abolished. This prompted us to perform a detailed study aiming at the identification of hemoglobin binding protein(s) present on the hepatocyte plasma membrane.

MATERIALS AND METHODS

Materials

Isolated rat cryopreserved hepatocytes were purchased from Invitrogen (Carlsbad, CA, USA). Rat and human hemoglobin were prepared from whole blood, as described previously (18). HepG2 cells were purchased from the Department of Embryology and Histology (Wroclaw Medical University, Poland).

Detection of hemoglobin receptors using the label transfer technique

SBED reagent was used to perform the label transfer technique. This compound is a cross-linking reagent containing two chemically-reactive groups and a biotin residue. Sulfonated N-hydroxysuccinimide ester reacts with primary amines and, thus, biotinylates the 'bait' protein (Hb). During the second step, photosensitive aryl azide moiety captures the target protein (putative Hb receptor). After reduction of the disulfide bridge, located on the N-linker side, the biotin label is transferred to the captured molecule. The released labeled receptor can be detected using streptavidin-peroxidase.

Human Hb was conjugated *via* amine groups to the tri-functional cross-linking agent sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido]-ethyl-1,3'-dithiopropionate (sulfo-SBED; Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The labeled hemoglobin is further referred to as SBED-Hb. Human hepatocellular carcinoma HepG2 cells were used as an *in vitro* model system for the experiment. The cells were routinely grown in MEM medium (Gibco/Life Technologies, Waltham, MA, USA) supplemented with 10% FCS (Lonza, Basel, Switzerland) and 1% L-glutamine-penicillin-streptomycin solution (Sigma). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. For experiments, the cells were harvested using 0.05% Trypsin-EDTA solution (Sigma-Aldrich, Germany) and seeded at a density of approximately 25 × 10⁴ cells/ml (12 ml) in Cellstar Petri dishes 145 × 20 mm (Greiner Bio One, Kremsmunster, Austria). The cells were grown to confluence over a period of 7 days and the media were changed every second day. On the day of the experiment, the cells ($1.6 × 10^7$) were washed three times in MEM without FCS and L-glutamine-penicillin-streptomycin solution and incubated with fresh medium without supplements for 1 hour.

For association of SBED-Hb with its cell receptors, the labeled protein (5 mg in 10 ml of growth medium) was added to the cells and the dishes were incubated in the dark at 37°C for 60 min. Unbound SBED-Hb was removed by washing with cold PBS. Cross-linking was achieved by exposure of the cells to long-wavelength UV light (365 nm) for 20 min at a distance of 5 cm on ice (except for the control dish). The cells were briefly washed two times with cold PBS, harvested by scraping in 3 ml of cold PBS with protease inhibitor cocktail and pelleted *via* centrifugation at 1000 × g for 5 min. Plasma membrane proteins were isolated using a ProteoExtract Native Protein Extraction Kit (Merck, Darmstadt, Germany), according to the manufacturer's instructions. The membrane proteins were analyzed by using NuPage 3 – 8% tris-acetate precast gels (Invitrogen, Carlsbad, CA) under reducing (100 mM dithiothreitol) or non-reducing conditions. The biotinylated proteins were detected *via* Western blotting method using horseradish peroxidase (HRP)-labeled streptavidin and an enhanced chemiluminescence reagent (Pierce, Rochester, IL, USA).

Isolation of membrane proteins

The total plasma membrane protein fraction (TM) and total surface protein fraction (TSM) of rat hepatocytes were isolated using a ProteoExtract ® Native Membrane Protein Extraction Kit and a Cell Surface Protein Isolation Kit (Merck, Darmstadt, Germany), respectively, according to the manufacturer's protocols.

Binding of hemoglobin to plasma membrane proteins by surface plasmon resonance (SPR)

The analysis was performed on a BIAcore 1000 analytical system, using a CM5 sensor chip (Biacore AB, Uppsala, Sweden) at 25 ± 0.1°C operating temperature with HBS (10 mM HEPES, 150 mM NaCl, and 0.05% surfactant P20, pH 7.4) as a mobile phase. An amine coupling kit was used to activate the carboxymethylated dextran surface of the sensor chip. Immobilization of the ligand was achieved *via* injection of TM (50 µl in 0.05 M sodium acetate buffer, pH 4.5 at 10 µl/min). Residual N-hydroxysuccinimide esters were inactivated with 1 M ethanolamine. For experiments, rat Hb (25 µM in HBS) was injected over the surface for the association phase. The surface was regenerated *via* subsequent washes with 10 mM HCl, 100 mM glycine and 10 mM NaOH.

Isolation of hemoglobin receptor with affinity chromatography

Rat Hb (5 mg) was immobilized *via* amine groups on 1 ml NHS-activated HiTrap Sepharose matrix (GE Healthcare, USA). The protein preparations (TM or TSM) were recirculated at 0.2 ml/min flow through an Hb-HiTrap Sepharose column equilibrated with PBS (pH 7.4), 1 mM $CaCl_2$, and 0.5% CHAPS for 2 hours. The column was washed with 30 ml PBS (pH 7.4), 1 mM $CaCl_2$, 0.5% CHAPS, 30 ml of the same buffer containing 0.5 M NaCl, and again with 10 ml of the first buffer. Bound proteins were eluted with 6 M urea in PBS pH 7.4 containing 0.5% CHAPS. For the control experiments, a ligand free column was used. Amine reactive sites on the bed were blocked with 1M ethanolamine for 1 hour, at 25°C. Collected 1-ml fractions were analyzed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using NuPage 3 – 8% tris-acetate

precast gels under reducing conditions (Invitrogen, Carlsbad, CA, USA). Protein bands were visualized with GELCODE blue stain reagent (Pierce Biotechnology, Rochester, IL, USA).

Protein identification via mass spectrometry

The receptor proteins isolated via affinity chromatography of the TSM fraction were identified using peptide mass fingerprinting analysis. After separation of proteins with SDS-PAGE followed by in-gel protein staining, the bands were excised and subsequently destained in 25% and 50% acetonitrile. The proteins were reduced and alkylated using 50 mM DTT and 10 mg/ml iodoacetamide. Digestion was performed using 10 ng/ml trypsin. The peptides were subsequently extracted using 10% TFA and 100% ACN, and dried. Before chromatography, the samples were resuspended in 2% ACN and 0.05% TFA, and centrifuged at 30,000 g for 30 min.

Peptides were separated using an Ultimate 3000RS LCnanoSystem (Dionex) chromatograph, and analyzed online using a microTOF-QII spectrometer (Bruker, Germany) equipped with an Appollo Source nano-ESI sprayer and a C18 guard column (Acclaim PepMap Nano Trap Column). Two percent ACN with 0.05% TFA was used as the mobile phase. Proper separation was performed on a 15 cm × 75 microns RP column (Acclaim PepMap 75 microns 100 Å Nano Series Column™) using a 40-minute gradient of 2 – 40% ACN with 0.05% FA. The spectrometer worked in standard DDA (date-dependent acquisition) mode, in which 3 precursors were subjected to the most intense fragmentation.

MS data were searched against the rodent sequences of the Swiss-Prot database using the Mascot search engine (v.3.0, Matrix Science, London, UK). The following search parameters were used: specificity of the enzyme-trypsin, allowed number of abandoned sites cut-1, modification-carbamidomethyl (Cys), variable modification-oxidation (Met), weight range-unrestricted, peptide mass tolerance of the precursor ± 20 ppm, ion fragment tolerance ± 0.05 Da.

Kinetics of hemoglobin binding to ATPB

Kinetics of hemoglobin-ATPB complex formation was analyzed via the fluorescence quenching technique using an LS50B spectrofluorimeter (Perkin Elmer, Waltham, MA, USA). All experiments were performed in PBS containing 2% glycerol in 5 × 5 mm cuvettes at 25°C. Excitation wavelength was set for 280 nm. The emission was scanned within a 300 – 400 nm range using emission and excitation slits of 10 and 5 nm, respectively.

In preliminary experiments, hemoglobin at a final concentration of 0.06 μM was incubated with ATPB (0.3 μM) and fluorescence emission was scanned every 5 min for a 1-hour period. The equilibrium of the complex Hb-ATPB formation was fully achieved within a 10-minute period.

For kinetic analysis, additions of hemoglobin (quencher) were made from stock solution (75 μM) to a cuvette (650 μl) containing 0.3 μM ATPB. After 10 min incubation, three subsequent scans were recorded. Scans were corrected by subtraction of the fluorescence signal recorded for the incubation buffer. Saturation binding curves were constructed using values recorded at 303 nm, the wavelength corresponding to the maximal emission of tyrosine residues. Dissociation constants (K_d) were evaluated via computerized non-linear regression analysis using GraphPad Prism software (GraphPad, USA) according to the one-site binding model corrected for ligand depletion effect (receptor concentration < 0.2 Kd) and quencher addition effect connected with intrinsic Hb fluorescence (19). A complementary experiment was performed with BSA as a non-binding ligand counterpart.

RESULTS

To achieve a preliminary characterization of the Hb binding components present in the hepatocyte membrane, we performed a study using the label transfer technique in hepatocyte derived cell line HepG2 (*Fig. 1*). Human Hb was conjugated to the
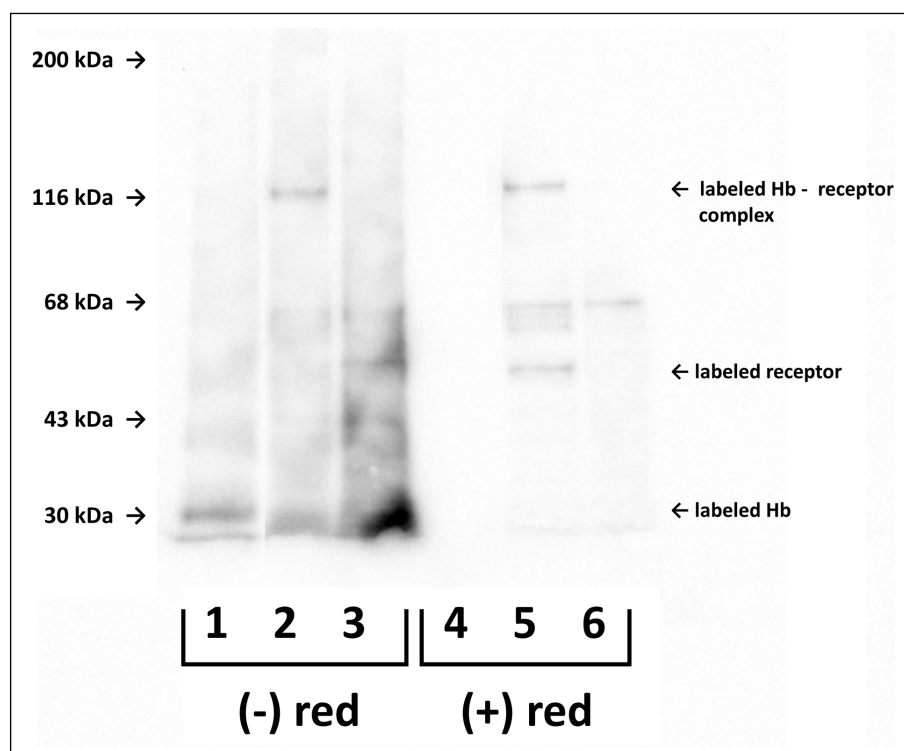
Fig. 1. Detection of the putative hepatocyte Hb receptor by using the biotin label transfer technique and Western-blotting. Hb was conjugated to the trifunctional cross-linking agent containing an amine linker, biotin label and photo-inducible aryl azide group (sulfo-SBED). SBED-Hb (bait protein) was incubated with the HepG2 cells. The putative Hb receptor (pray protein) was captured by photoactivation of the aryl azide moiety linked via cleavable disulfide bond to Hb). The biotin residue of SBED-Hb (lane 1) is fully split under reducing conditions (lane 4). The complex SBED-Hb-putative receptor is seen as ~120 kDa band (lane 2). After reduction of the disulfide bridge, the biotin label is transferred to the captured ~50 kDa Hb receptor (lane 5). The bands are not seen in the control experiment without a photoactivation step (lane 3 and 6). Biotin was visualized using an HRP-streptavidin conjugate and chemiluminescent detection.
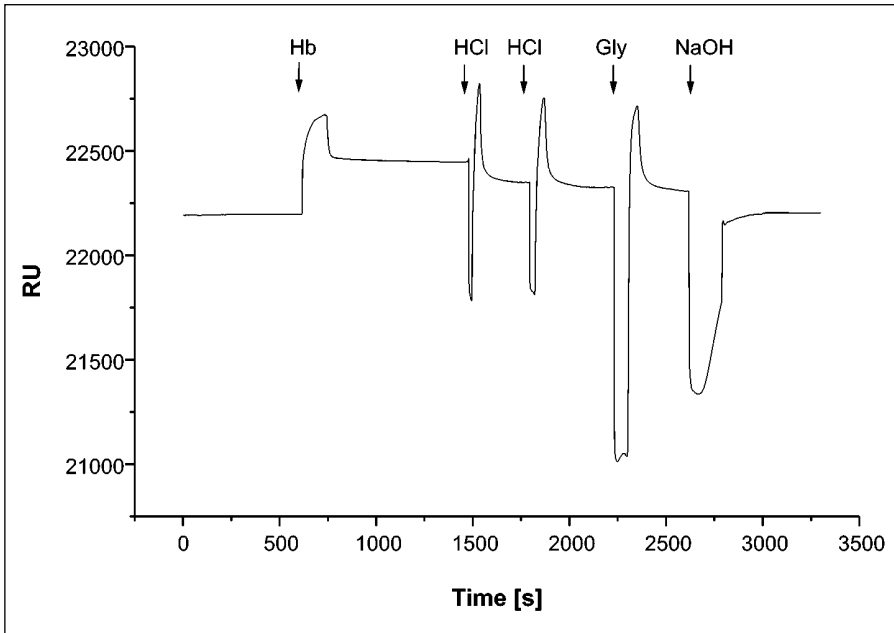
Fig. 2. Characterization of hemoglobin binding to the hepatocyte membrane protein fraction by SPR. Hb (analyte) was passed over total hepatocyte membrane proteins (ligands) immobilized on a sensor chip. Associated Hb was released by acidic treatment (10 mM HCl) in only ca. 30%. Mild alkaline treatment (100 mM Gly) did not support further release. Full dissociation was achieved under strong alkaline conditions (10 mM NaOH). The SPR signal is presented as the mass equivalent response units (RU).
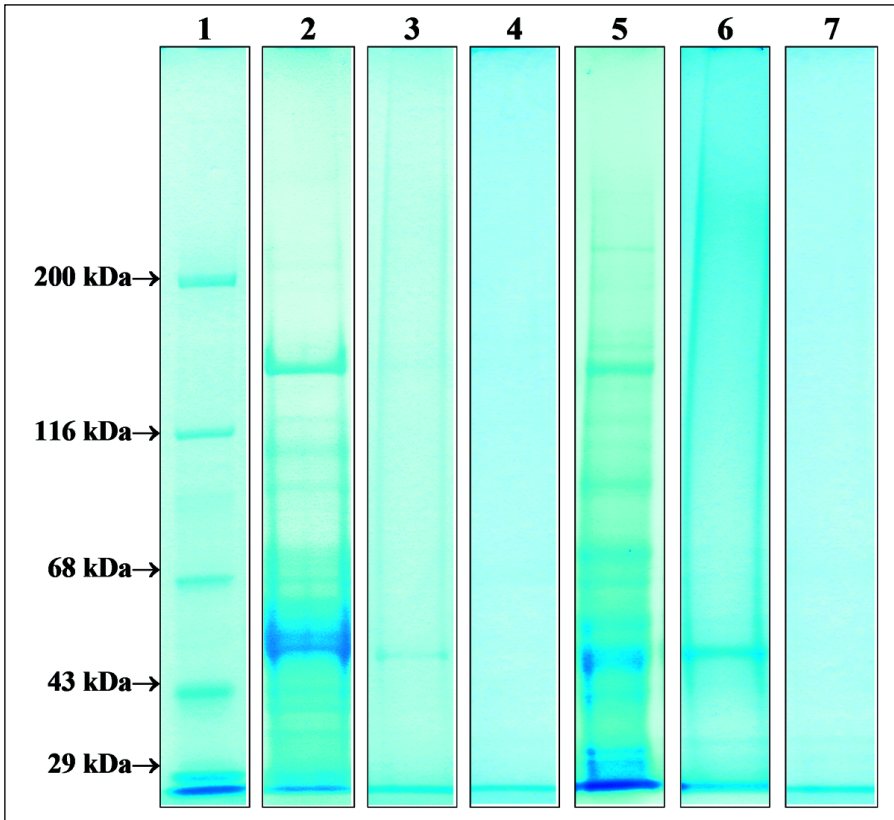
Fig. 3. Affinity chromatography of hepatocyte plasma membrane proteins. Chromatography was performed on a bed with (lanes 3, 6) or without (lanes 4, 7) immobilized hemoglobin. (lane 1) molecular weight markers; (lane 2) total plasma membrane proteins; (lanes 3, 4) affinity isolated protein fraction extracted from total plasma membrane proteins; (lane 5) total surface protein fraction; (lanes 6, 7) affinity isolated protein fraction extracted from total surface protein fraction. The fractions were analyzed in SDS-PAGE. Gels were stained using colloidal Coomassie Brilliant Blue.

trifunctional cross-linking agent containing an amine linker, biotin label and photoinducible aryl azide group (sulfo-SBED). Under non-reducing conditions, SBED-Hb conjugate could be easily detected using a streptavidin-peroxidase reagent. In the main, a hemoglobin dimer band was visible (Lane 1). Complete splitting of the biotin moiety occurred after reduction (Lane 4). The cells were first incubated with SBED-Hb at 37°C for 60 min in the dark. Following incubation, unbound SBED-Hb was removed, the cells were washed, and exposed to long-wavelength UV light in order to activate the aryl azide moiety and, consequently, cross-link the Hb-receptor complex. In the control experiment, the photo-induction step was omitted. Transmembrane protein fractions were isolated from the cells and analyzed via Western-blotting method. A 120 kDa band corresponding to the cross-linked Hb-receptor complex was seen under non-reducing conditions (Lane 2). Under reducing conditions, the band had lower intensity and a new ca. 50 kDa band appeared, corresponding to the labeled receptor (Lane 5). This was consistent with the splitting of the disulfide bridge in the cross-linked complex and the transfer of the biotin label to the receptor molecule. The shift in molecular mass coincides with the mass of the released tetrameric Hb (66 kDa). Neither

Table 1. Hb receptor proteins isolated by affinity chromatography and identified by mass spectrometry.

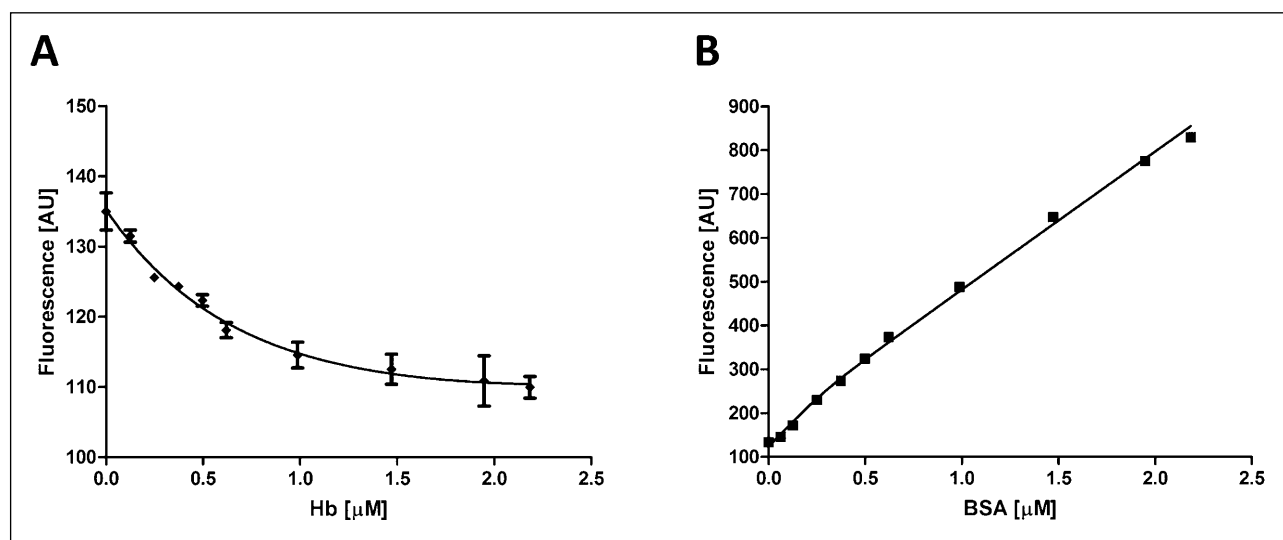| Protein identified	Mr [kDa]	Score	# Peptides	S.C. [%]
ATPase alpha subunit (ATPA_RAT)	59.7	1759	20	52.8
ATPase beta subunit (ATPB_RAT)	56.3	1745	24	69.4
Trifunctional enzyme subunit beta (ECHB_RAT)	51.4	420	7	29.7
Cytochrome P450 2C23 (CP2CN_RAT)	56.4	281	6	16.2
Fatty aldehyde dehydrogenase (AL3A2_RAT)	54.0	224	4	11.6

Fig. 4. Quenching of ATPB intrinsic fluorescence by Hb. Increasing amounts of pure hemoglobin (A) or BSA (B) in the minimal volume were added to a reaction cuvette containing ATPB. BSA was used as a non-binding protein counterpart (negative control). Fluorescence emission at 303 nm (excitation wavelength of 280 nm) was recorded. Additive quenching of fluorescence is seen with Hb addition indicating complex formation, while no quenching is observed with BSA addition.

120 kDa nor 50 kDa bands were present in the preparations obtained from the control experiment (Lanes 3 and 6). The bands seen in lanes 5 and 6 in the range of 65 – 70 kDa presumably represent non-specific binding of streptavidin to a non-identified cell membrane component.

Next, we established the optimal conditions for isolation of the hepatocyte hemoglobin receptor in the SPR study. Total protein membrane fractions from the isolated rat hepatocytes were used. The proteins were coated covalently on a sensor chip and rat hemoglobin was passed over as an analyte (Fig. 2). Substantial binding of hemoglobin occurred, as was seen by a baseline shift of about 250 RU. The affinity of the interaction was in the nanomolar range (Kd = 9.14×10^{-7} M) when the curves were analyzed according to the one-site binding model. The strength of the interaction was substantial enough to approach the isolation of the receptor using affinity chromatography. The binding could not be completely released in acidic conditions (10 mM HCL, 100 mM Gly). Additional basic treatment (10 mM NaOH) was needed in order to fully dissociate the complex. Therefore, we decided to use a strong chaotropic agent-urea-as an eluent in affinity chromatography.

Two different hepatocyte protein preparations were employed for isolation of the receptor: (i) the total plasma membrane protein fraction, and (ii) the surface protein fraction. The fractions were passed over the column containing the hemoglobin-matrix and the eluates were analyzed by using SDS-PAGE. Both fractions yielded the same major band,

corresponding to a molecular mass of about 50 kDa (Fig. 3). There were no bands visible in the control experiments using a bed without hemoglobin (Fig. 3).

The results from the label transfer experiment and affinity chromatography were consistent, and indicated that the putative Hb receptor is a protein with a molecular mass of ca. 50 kDa. The purified protein was subjected to peptide mass fingerprinting analysis for identification. Two predominant components were detected in the preparation: F1-ATPase subunit alpha (ATPA) and beta (ATPB). The details of the analysis are presented in Table 1. Both the signal score (1745 – 1759 AU) and the sequence coverage (53 – 69%) for these two proteins were relatively high, confirming the validity of the analysis. The other minor components of the fraction, due to their relatively poor scores, can be considered non-specific contaminations, presumably originating from the endoplasmic reticulum.

In a further study, the binding of Hb to F1-ATPase was verified using recombinant rat ATPB. The purity of the protein was verified via SDS-PAGE (not shown). There was no contamination in the preparation. The interaction of purified proteins was examined using the fluorescence quenching technique. Increasing amounts of pure hemoglobin or BSA in a minimal volume were added to a reaction cuvette containing 0.3 µM ATPB or binding buffer alone (control). BSA was used as a non-binding protein counterpart. Since ATPB does not contain tryptophan residues, the intrinsic fluorescence of tyrosine
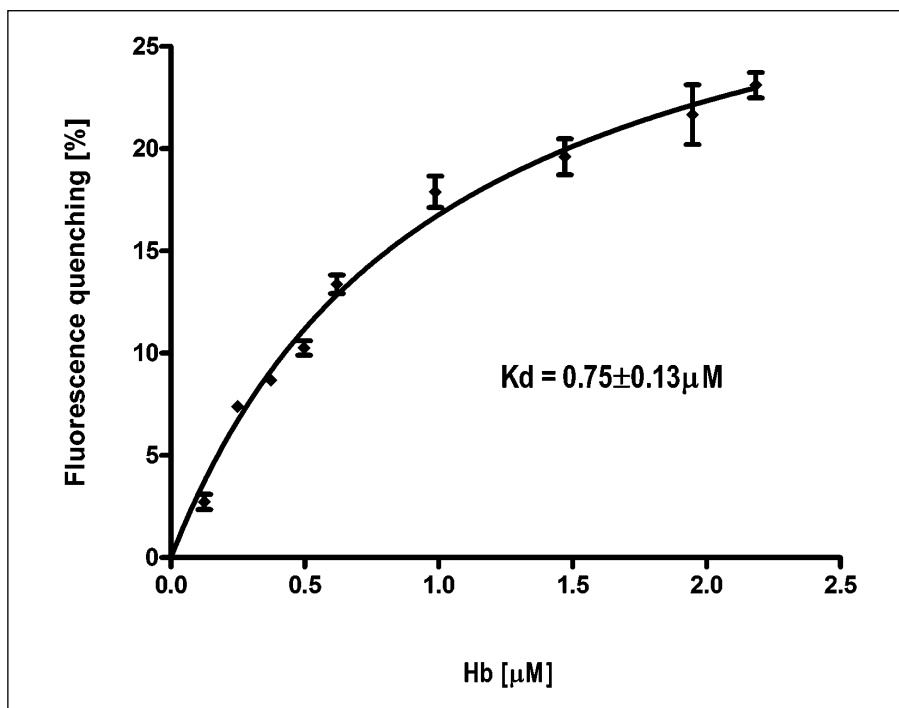
Fig. 5. Saturation binding curve of hemoglobin binding to F1-ATPase beta subunit. Association was examined using the fluorescence quenching technique. Increasing amounts of pure Hb in the minimal volume were added to a reaction cuvette containing 0.3 μmol ATPB. K_d was evaluated by non-linear regression according to the one-site binding model. The data are presented as mean $\pm$ standard errors from three measurements.

residues was recorded at an excitation wavelength of $\lambda_{ex} = 280$ nm. Fluorescence emission spectra were recorded in the range from 300 nm to 400 nm. The values recorded at 303 nm (the maximum emission for tyrosine residues) were extracted for further analyses. There was an additive fluorescence signal rise when increasing the concentration of pure hemoglobin in the binding buffer, indicating that the inner quenching effect did not affect the measurements. In contrast, significant fluorescence quenching was observed when increased amounts of hemoglobin was added to ATPB (*Fig. 4A*). Such a fluorescence shift is in accordance with changes in the environment of tyrosine residues due to conformational changes in the course of complex formation between the two proteins (dynamic-static quenching). As in the case of hemoglobin, increasing the BSA concentration in binding buffer alone gave rise to the fluorescence emission signal in a dose-dependent manner. However, no quenching of fluorescence was observed when BSA was added to ATPB, indicating no complex formation (*Fig. 4 B*). Therefore, the interaction between Hb and ATPB seems to be relatively specific.

The kinetics of the complex dissociation were evaluated via non-linear regression analysis. Hb binding to ATPB exhibited saturable binding kinetics (*Fig. 5*). Fitting the curve according to the one-site binding model yielded K_d of 7.5×10^{-7} M. The affinity was in close accordance with the data obtained with a crude membrane protein fraction using SPR ($K_d = 9.1 \times 10^{-7}$ M).

DISCUSSION

Uptake of hemoglobin by hepatocytes and the presence of specific binding sites on the hepatocyte membrane have been repeatedly reported over the last half century. However, research has so far failed to identify the specific Hb receptor in these cells.

In the present study, we have isolated the hepatocyte Hb receptor on the Hb matrix *via* affinity chromatography using two different membrane protein preparations obtained from isolated rat hepatocytes. The first method, based on specific non-detergent extraction, has previously been reported to yield pure membrane proteins, free of other subcellular protein contaminations, and has proved to be superior than the five currently used protocols (20). The second method employed surface biotinylation of whole cells as the first step and subsequent streptavidin-based extraction, ensuring that only surface plasma membrane proteins are captured. Both preparations yielded the same two major protein components. The proteins were identified *via* mass spectrometry as F1-ATPase alpha (ATPA) and beta (ATPB) subunits. It is well documented that F1-ATPase is expressed in the plasma membrane, in addition to its mitochondrial expression. Both the ectopic and mitochondrial forms seem to be identical as regard to the structure and stoichiometry of subunits (21, 20). The plasma membrane expression of F1-ATPase is linked to different biological activities, such endocytosis/regulation of HDL uptake by hepatocytes, endothelial cell proliferation, and antitumor activity of T lymphocytes. Currently, only a few ligands for the receptor have been identified, i.e. apoA1, MHC1 antigen, angiostatin, amyloid beta peptide and juvenile hormone binding protein. Although it is known from recent studies that the whole F0F1 complex is present in the plasma membrane, the receptor activities are attributed to the F1 part of the complex (22, 23). Consistently, we observed hemoglobin binding to both components of the F1 complex (alpha and beta subunits).

Using pure recombinant rat ATPB and Hb, we estimated the dissociation constant for the complex ($K_d = 7.5 \times 10^{-7}$ M). The value was close to that obtained using the total protein membrane fraction of hepatocytes analyzed via the SPR method ($K_d = 9.1 \times 10^{-7}$ M) and that reported in our previous study (10) using EDTA-prepared hepatocytes ($K_d = 1.2 \times 10^{-6}$ M). As previously reported (24), the concentration of free hemoglobin in normal plasma fell into the same range (~0.5×10^{-6} M). Therefore, the interaction of hemoglobin and F1-ATPase may be relevant for the hepatic uptake of the protein in physiological state. The other Hb uptake system involving megalin/cubilin tandem receptors, operating with similar affinity ($K_d = 1.7 - 4.1$

$\times 10^{-6}$ M), has proved to be efficient for complete extraction of the protein in the renal proximal tubule (4). In hemolytic disorders the concentration of hemoglobin can be elevated several times. Under these circumstances, rapid complex formation can be expected. Thus, the pathway might be responsible for the massive loading of hemoglobin into the cells, and consequently liver toxicity observed during extensive hemolysis or hemoglobin-based blood substituent infusions.

It seems that ectopic F1-ATPase does not perform the role of classical scavenger receptor, but rather it is a regulatory protein that induces endocytic processes in the hepatocyte. In the case of HDL/ApoA1, the enzyme acts in concert with the P2Y$_{13}$ receptor to facilitate endocytic uptake of these ligands. Hydrolysis of ATP to ADP by F1-ATPase stimulates the P2Y13 receptor signaling, dependent on small RhoA GTPase that results in the uptake of the HDL particles. We observed similar characteristics for Hb uptake by cultured isolated hepatocytes in our previous study. Internalization of Hb was dependent on ATP and stimulated by GTP addition to the medium (10). The exact molecular mechanism of F1-ATPase driven endocytosis has not yet been revealed. It is not known whether ectopic ATPase undergoes internalization and recycling. Also, adaptor proteins associated with the receptor have not been identified. Since the receptor seems to be associated with membrane lipid rafts, it can be speculated that a clathrin-independent mode of endocytosis underlies this phenomenon.

Due to the significant prevalence of hemolytic diseases, this finding may be very important for future studies into the pathogenesis of acute hemolysis complications. Our results may also be important for the development of safe blood substitutes based on hemoglobin for use in medical intervention.

In addition, due to the abundant presentation of ectopic F1-ATPase on hepatocyte cells, the receptor could be a very promising target molecule for specific delivery of drugs in treatment of liver diseases. Antioxidant, antiinflamatory and antiapoptotic agents seem to be effective in attenuation of liver injure in several experimental models of chemical intoxication and hepatitis, and could be possibly directed to hepatocyte cells *via* this interaction (25). On the other hand, maneuvers targeting proteins associated with endocytosis and autophagy are considered as new strategies in anticancer therapy (26, 27).

In the present study, we have identified for the first time the fact that ectopic F1-ATPase is the receptor for hemoglobin on the hepatocyte plasma membrane. We suggest that the receptor is involved in hemoglobin uptake by hepatocytes. Since ectopic F1-ATPase is not internalized itself, it seems that its role is limited to a regulatory function in the endocytic processes. Further studies are needed to explain the exact role of the receptor in physiological and pathological states.

Abbreviations: ACN, acetonitrile; ATPA, F1-ATPase subunit alpha; ATPB, F1-ATPase subunit beta; DTT, dithiotreitol; Hb, hemoglobin; Hp, haptoglobin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPR, suface plasmon resonance; Sulfo-SBED, sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido]-ethyl-1,3'-dithiopropionate; TFA, trifluoroacetic acid.

Conflict of interests: None declared.

REFERENCES

1. Lim SK, Ferraro B, Moore K, Halliwell B. Role of haptoglobin in free hemoglobin metabolism. *Redox Rep* 2001; 6: 219-227.
2. Schaer DJ, Buehler PW, Alayash AI, Belcher JD, Vercellotti GM. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. *Blood* 2013; 121: 1276-1284.
3. Zager RA. Rhabdomyolysis and myohemoglobinuric acute renal failure. *Kidney Int* 1996; 49: 314-326.
4. Gburek J, Verroust PJ, Willnow TE, *et al*. Megalin and cubilin are endocytic receptors involved in renal clearance of hemoglobin. *J Am Soc Nephrol* 2002; 13: 423-430.
5. Goldfischer S, Novikoff AB, Albala A, Biempica L. Hemoglobin uptake by rat hepatocytes and its breakdown within lysosomes. *J Cell Biol* 1970; 44: 513-529.
6. Seki K, Shindo M, Sawasaki Y, Nakajama H, Ishikawa H, Yamada E. Histological studies on hemoglobin degradation. *Proc Japan Acad* 1979; 55: 502-507.
7. Kino K, Tsunoo H, Higa Y, Takami M, Hamaguchi H, Nakajima H. Hemoglobin- haptoglobin receptor in rat liver plasma membrane. *J Biol Chem* 1980; 25: 9616-9620.
8. Kino K, Tsunoo H, Higa Y, Takami M, Nakajima H. Kinetic aspects of hemoglobin- haptoglobin receptor interaction in rat liver plasma membranes, isolated liver cells and liver cells in primary culture. *J Biol Chem* 1982; 257: 4828-4833.
9. Kino K, Mizumoto K, Watanabe J, Tsunoo H. Immunohistochemical studies on hemoglobin-haptoglobin and hemoglobin catabolism sites. *J Histochem Cytochem* 1987; 35: 381-386.
10. Zuwala-Jagiello J, Osada J. Internalization study using EDTA-prepared hepatocytes for receptor-mediated endocytosis of haemoglobin-haptoglobin complex. *Int J Biochem Cell Biol* 1998; 30: 923-931.
11. Osada J, Gburek J, Zuwala-Jagiello J. Membranous receptors of haemoglobin in liver and kidney. *Diagn Lab* 1999; 35: 409-412.
12. Kristiansen M, Graversen JH, Jacobsen C, *et al*. Identification of the haemoglobin scavenger receptor. *Nature* 2001; 409: 198-201.
13. Schaer DJ, Schaer CA, Buehler PW, *et al*. CD163 is the macrophage scavenger receptor for native and chemically modified hemoglobins in the absence of haptoglobin. *Blood* 2006; 107: 373-380.
14. Van Gorp H, Delputte PL, Nauwynck HJ. Scavenger receptor CD163, a Jack-of-all-trades and potential target for cell-directed therapy. *Mol Immunol* 2010; 47: 1650-1660.
15. Etzerodt A, Moestrup SK. CD163 and inflammation: biological, diagnostic, and therapeutic aspects. *Antioxid Redox Signal* 2013; 18: 2352-2363.
16. Etzerodt A, Kjolby M, Nielsen MJ, Maniecki M, Svendsen P, Moestrup SK. Plasma clearance of hemoglobin and haptoglobin in mice and effect of CD163 gene targeting disruption. *Antioxid Redox Signal* 2013; 18: 2254-2263.
17. Fagoonee S, Gburek J, Hirsch E, *et al*. Plasma protein haptoglobin modulates renal iron loading. *Am J Pathol* 2005; 166: 973-983.
18. Gburek J, Osada J. Hemoglobin binding sites on renal brush-border membranes. *Biochimie* 2000; 82: 1135-1142.
19. Groemping Y, Hellmann N. Spectroccopic methods for determination of protein interaction. In: Current Protocols in Protein Science, Coligan JE *et al*. (eds.). Wiley and Sons Inc. 2005, Suppl. 39, Unit 20.8.
20. Bunger S, Roblick UJ, Habermann JK. Comparison of five commercial extraction kits for subsequent membrane protein profiling. *Cytotechnology* 2009; 61: 153-159.

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21. Bae TJ, Kim MS, Kim JW, *et al*. Lipid raft proteome reveals ATP synthase complex in the cell surface. *Proteomics* 2004; 4: 3536-3548.

22. Rai AK, Spolaore B, Harris DA, Dabbeni-Sala F, Lippe G. Ectopic F0F1 ATP synthase contains both nuclear and mitochondrially-encoded subunits. *J Bioenerg Biomembr* 2013; 45: 569-579.

23. Vantourout P, Radojkovic C, Lichtenstein L, Pons V, Champagne E, Martinez LO. Ecto-F1-ATPase: a moonlighting protein complex and an unexpected apoA-I receptor. *World J Gastroenterol* 2010; 16: 5925-5935.

24. Fairbanks VF, Ziesmer SC, O'Brien PC. Methods for measuring plasma hemoglobin in micromolar concentration compared. *Clin Chem* 1992; 38: 132-140.

25. Toton E, Lisiak N, Sawicka P, Rybczynska M. Beclin-1 and its role as a target for anticancer therapy *J Physiol Pharmacol* 2014; 65: 459-467.

26. Hu CT, Wu JR, Wu WS. The role of endosomal signaling triggered by metastatic growth factors in tumor progression. *Cell Signal* 2013; 25:1539-1545.

27. Galaly SR, Ahmed OM, Mahmoud AM. Thymoquinone and curcumin prevent gentamicin-induced liver injury by attenuating oxidative stress, inflammation and apoptosis. *J Physiol Pharmacol* 2014; 65: 823-832.

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