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Hemoglobin interacting proteins and implications of spectrin hemoglobin interaction

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ABSTRACT

In this report we have analyzed interacting partners of hemoglobin inside erythrocyte and sought possible implications of hemoglobin–spectrin interaction. Our list of identified cytosolic hemoglobin interacting proteins includes redox regulators like peroxiredoxin-2, Cu–Zn superoxide dismutase, catalase, aldehyde dehydrogenase-1, flavin reductase and chaperones like HSP70, α-hemoglobin stabilizing protein. Others include metabolic enzymes like carbonic anhydrase-1, selenium binding protein-1, purine nucleoside phosphorylase and nucleoside diphosphate kinase. Additionally, various membrane proteins like α and β spectrin, ankyrin, band3, protein4.1, actin and glyceraldehyde 3 phosphate dehydrogenase have been shown to interact with hemoglobin. Our result indicates that major membrane skeleton protein spectrin, that also has a chaperone like activity, helps to fold the unstable alpha-globin chains *in vitro*. Taken together our results could provide insight into a protein network evolved around hemoglobin molecule inside erythrocyte that may add a new perspective in understanding the hemoglobin function and homeostasis.

1. Introduction

Biological processes are mostly carried out through specific protein–protein interactions [1]. Identification and comprehension of these interactions are essential for rationalization of structure–function relationships and exploration of their roles in the progress of related diseases. With the tremendous advancement in the field of mass spectrometry, escalating work has been done in the area of erythrocyte proteomics [2–9]. Till date about 2289 non-redundant proteins had been identified in the erythrocyte [10]. This wealth of knowledge gives a firm basis for studying protein–protein interactions within the erythrocytes. Up till now, mainly *in silico* protein–protein interactions inside erythrocyte have been predicted through bioinformatics [10–12] and only a small number of these have been validated by large scale experiments [13,14].

Hemoglobin (Hb), an iron-containing oxygen-transport protein, is the most abundant protein of erythrocytes [12]. Previously, carbonic anhydrase-1(CA-1) was shown to interact with Hb [13]. Alpha hemoglobin stabilizing protein (AHSP) also interacts with free alpha globin chains [15]. Peroxiredoxin-2 (Prdx2) was shown to interact with Hb in mouse erythrocytes and protects it from oxidative stress [16]. In human, Prdx2 also interacts with hemoglobin and forms high molecular weight complexes under stressed condition [17]. Among erythrocyte membrane proteins, band3 was found to interact with deoxyhemoglobin with plausible implications [18]. The major membrane skeleton protein of erythrocytes, spectrin was also earlier shown to interact with Hb and globin chains [19], but the functional significance of these interactions was not studied in details. Association of Hb with multiple proteins could possibly play modulating role in the pathophysiology of hemoglobinopathies like thalassemia.

There is a lack of systematic studies on proteins interacting with Hb and their possible involvement in hemoglobin function. Towards this goal we have analyzed the proteins directly interacting with Hb and found that several cytosolic redox regulators, chaperones and metabolic enzymes along with key membrane skeleton proteins interact with Hb. We believe that these results might shed some light into hemoglobin function and homeostasis inside red cell.

2. Materials and methods

2.1. Materials

Blood samples from normal healthy volunteers (4 male and 2 female candidates, aged between 24 and 28 years) were obtained within the institute, with informed written consent following the guidelines of the Institutional Animal & Bio ethics Committee of Saha Institute of Nuclear Physics. The healthy normal population has not suffered from any hematological disorder.

PVDF membrane and molecular cut off filters were purchased from Millipore (Billerica, MA, USA). Sulphopropyl sephadex (SP sephadex) cation exchange column, percoll, α-Cyano-4-hydroxycinnamic acid (CHCA), and guanidium hydrochloride (GdnCl) were purchased from Sigma (St. Louis, MO, USA), and electrophoresis reagents were obtained

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from Bio-Rad (Hercules, CA, USA). Sequencing grade trypsin was purchased from Promega (Madison, WI, USA). Ni-NTA beads, western ECL reagent, and in-gel trypsin digestion reagents were obtained from Pierce Biotechnologies (Bedford, MA, USA). Antibodies for alpha-globin, HSP70, Prdx2, ST13 and SOD-1 were obtained from Abcam (Cambridge, MA, USA), and for catalase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents, if not mentioned otherwise, were purchased locally and were of analytical grade.

2.2. Erythrocyte processing and spectrin purification

Red blood cells were purified to >99.5% purity using 75% percoll method as described earlier [20,21]. We performed hemoglobin concentration measurements as well as analyzed the hemoglobin variant profiles using Variant™ HPLC (Bio-Rad, Hercules, CA, USA). The hemoglobin concentration ranges from 12 to 14 mg/ml. In the HPLC profile the HbA2 window is <3% and HbF level around 0.5%.

Purified erythrocytes were washed with PBS and subjected to hypotonic lysis in a buffer containing 5 mM sodium phosphate (pH 8.0), 1 mM EDTA 20 mg/ml PMSF. The cytosol was separated from the membrane by centrifugation at 30,000 *g* at 4 °C and membrane fraction was subsequently washed 5 times to remove the residual hemoglobin to obtain white ghost (WG). Dimeric spectrin was purified from WG at 37 °C following the previous protocol [22]. Our basic workflow for identification of potential hemoglobin interacting partners is represented in Fig. 1.

2.3. Hemoglobin depletion

The cytosol was separated from the membrane by centrifugation and subjected to hemoglobin depletion described earlier [20]. Briefly, hemoglobin A were kept bound on the SP-sephadex matrix at pH 6.7 while other non-hemoglobin proteins were obtained in the flow through.

2.4. Hemoglobin and globin chain purification

Hemoglobin was isolated from packed erythrocytes by osmotic lysis using 5 volumes of 1 mM Tris, pH 8.0, at 4 °C for 1 h. The hemoglobin mixture was purified by gel filtration on Sephadex G-100 column (30 × 1 cm) in a buffer containing 5 mM Tris, 50 mM KCl, pH 8.0. Individual globin chains were prepared from the purified hemoglobin following our previous protocol [23].

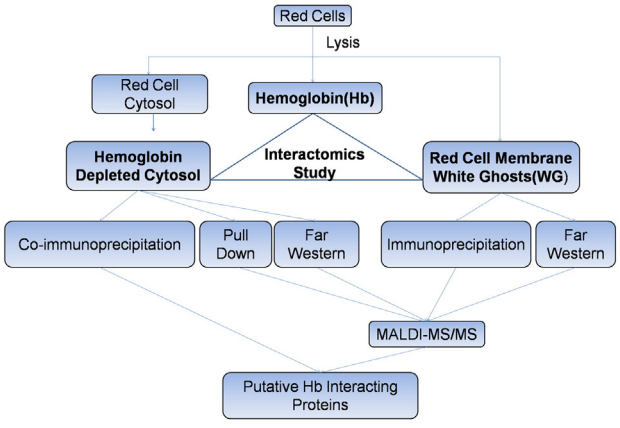

Fig. 1. Workflow for identification of potential hemoglobin interacting partners. Flow chart showing different methods used to identify the putative hemoglobin interacting proteins from erythrocyte cytosol as well as membrane fraction (see Materials and methods section for full details).

2.5. Pull-down experiment

Commercially available Ni-NTA columns, used to purify His tagged fusion proteins have been employed to trap hemoglobin molecule [24]. We have used this Ni-NTA pull down technique using the 'ProFound™ Pull-Down PolyHis Protein:Protein Interaction Kit' (Pierce Biotechnologies, Bedford, MA) to identify the hemoglobin interacting partners. Purified Hb was used as bait and pre-cleared (previously equilibrated with the resin) Hb depleted cytosol was used as prey in these experiments. Purified Hb was bound to the Ni-NTA based resin column for 1 h and prey protein pool was incubated for 4 h with the Hb-bound resin. For 200 μg of bait protein, 800 μg of the prey protein was used. This experiment was repeated (n = 3) to attain reproducible pattern of spots in 2D gel. Every time a control experiment was done by following the same pull-down protocol in absence of bait protein. After discarding the flow-through and subsequent washing with lysis buffer containing 20 mM imidazole, the interaction complex as well as the control fraction was eluted using 200 mM imidazole.

2.6. Two dimensional gel electrophoresis

The first dimension, isoelectric focusing (IEF), was performed in a 7-cm IPG Strip (Bio-Rad, Hercules, CA) with a pH 4–7 gradient using PROTEAN IEF Cell (Bio-Rad, Hercules, CA) at 20 °C. IPG strip rehydration and isoelectric focusing was performed overnight in a rehydration buffer (8 M Urea, 4% CHAPS, 0.04% Bromophenol Blue 2% Pharmalyte 3–10 NL, 8 M Urea, and 2% dithiothreitol-DTT). After reduction and alkylation, the proteins separated by IEF were further separated by 12% or 15% SDS PAGE at constant current (30 mA) and room temperature till the dye font reaches the bottom. The gels were post stained with Coomassie brilliant blue (Molecular Probes, Invitrogen, Eugene, OR) according to the manufacturer's protocol for identification of protein spots by mass spectrometry.

2.7. 1D and 2D far western blotting

Peroxidase activity of Hb was exploited for identification of the interacting partners of Hb by modified far western technique. The hemoglobin depleted erythrocyte cytosol was subjected to 2D immunoblotting whereas the WGs were subject to 1D immuno-blotting and then probed with purified Hb. After subsequent washing to avoid non-specific binding the blots were developed with enhanced chemiluminescence (ECL) substrates (Pierce, Bedford, MA). The signals in the blot correspond to the position of the Hb interacting proteins in the 1D or 2D map. The proteins were identified directly from the Coomassie blue after stained PVDF membrane, by mass spectrometry.

2.8. Immunoprecipitation

Immunoprecipitation experiments were performed to identify the Hb interacting proteins present in erythrocyte membrane fraction. In each experiment using 500 μg of the unwashed erythrocyte membrane proteins (that still partially retains Hb), the Hb and its interacting partners were immuno-precipitated with anti-α globin antibody (Abcam, Cambridge, MA). Protein A/G bead was used to track the whole immune complex and a negative control was set using mouse anti-IgG primary antibody (along with protein A/G bead) to rule out non specific binding of the proteins with either A/G beads or antibody Fc region. After running 1D SDS-PAGE the interacting proteins were identified by mass spectrometry.

2.9. MALDI TOF/TOF mass spectrometry

The protein spots from 1D/2D gels/PVDF membranes were digested with trypsin according to Shevchenko and co-workers [25] with minor modifications using Trypsin Gold from Promega (Madison, WI, USA).

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