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Identification and characterization of the hemoglobin-binding domain of hemoglobin receptor in *Leishmania*

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Leishmania **internalize hemoglobin (Hb)** ***via*** **a specific receptor (HbR) for their survival. To identify the Hb-binding domain of HbR, we cloned and expressed several truncated proteins of HbR and determined their ability to bind Hb. Our findings reveal that 90% of Hb-binding activity is retained in $HbR^{41–80}$ in comparison with $HbR^{1–471}$. We synthesized a 40 amino acid peptide (SSEKMKQLTMYMIHEMVEGLEGRPSTVRMLPSFVYTSDPA) corresponding to $HbR^{41–80}$ and found that it specifically binds Hb. Subsequently, we found that the $HbR^{41–80}$ peptide completely blocks Hb uptake in both promastigote and amastigote forms of *Leishmania* and, thereby, inhibits the growth of the parasite. These results demonstrate that $HbR^{41–80}$ is the Hb-binding domain of HbR, which might be used as a potential therapeutic agent to inhibit the growth of *Leishmania*.**

Keywords: domain; endocytosis; hemoglobin; *Leishmania*; receptor

Leishmaniasis represents a complex of diseases, such as visceral leishmaniasis (VL), mucocutaneous leishmaniasis, and cutaneous leishmaniasis (CL), caused by different species of *Leishmania* [1]. Among these, VL caused by *Leishmania donovani* and *Leishmania infantum* is fatal if untreated [2]. About 12 million people are affected by leishmaniasis and about 350 million people are estimated at risk of disease [3,4]. In addition, CL is endemic in more than 70 countries worldwide. Another major problem is zoonotic VL (ZVL), caused by *L. infantum* affecting dogs and humans [5,6]. As dogs are the major reservoirs for *L. infantum*, they play significant impact on human disease in many European countries [7]. In addition, drugs used in the chemotherapy of leishmaniasis are very toxic and expensive, and no licensed vaccine is available [8,9]. Besides, frequent resistance to different drugs is a major problem [10]. Thus, the major thrust is to identify a common chemotherapeutic target across different species of *Leishmania* for controlling various forms of leishmaniasis.

Leishmania is an auxotroph for heme as the parasite is unable to synthesize it [11]. Therefore, the heme acquisition process in the parasite can be a potential target for novel drugs [12]. Previously, we have shown that hemoglobin (Hb) is internalized by *Leishmania* through a specific receptor [13,14], HbR, *via* a clathrin-dependent process [15]. Subsequently, we have shown that HbR is a hexokinase with an N-terminal extracellular Hb-binding domain ($HbR^{1–126}$) and a C-terminal cytoplasmic domain ($HbR^{270–471}$) [14]. In addition, we have found that internalized Hb is targeted to the lysosomal

Abbreviations
CL, cutaneous leishmaniasis; Hb, hemoglobin; HbR, Hb receptor; Ld, *Leishmania donovani*; VL, visceral leishmaniasis.

compartment by a Rab5- and Rab7-dependent endocytic pathway where it is degraded to generate intracellular heme, which is used by the parasites [16,17]. Thus, this receptor system plays a very significant role in the survival of *Leishmania* [15,17]. Therefore, inhibiting Hb endocytosis in *Leishmania* can be a potential therapeutic target. This is supported by the fact that HbR is a novel vaccine candidate for Leishmaniasis [18]. However, Hb-binding domain of HbR is not yet characterized. Thus, it will be important to identify the minimal Hb-binding domain of the receptor which can be used for blocking Hb endocytosis to arrest the growth of the parasites.

In the present investigation, we have identified the Hb-binding domain of the Hb receptor, comprising of 40 amino acid residues which retain complete Hb-binding activity. Finally, we have shown that this 40 amino acid peptide blocks Hb uptake in the parasites and, thereby, significantly inhibits the growth of *Leishmania*.

Materials and methods

Materials

Unless otherwise stated, all reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Platinum high fidelity *Taq* polymerase and restriction enzymes were purchased from Invitrogen (Carlsbad, CA, USA) and Promega Life Science (Madison, WI, USA), respectively. Glutathione sepharose 4B beads, protein markers (RPN756 and RPN800), and ECL reagents were obtained from Amersham Bioscience (Amersham, UK). Alexa Fluor-594 succinimidyl ester and Prolong antifade were obtained from Molecular Probes (Eugene, OR, USA). *N*-hydroxysuccinimido-biotin (NHS-biotin) and avidin–horseradish peroxidase (avidin–HRP) were purchased from Vector Laboratories (Burlingame, CA, USA). Hb was biotinylated by using NHS-biotin. All other reagents used were of analytical grade.

Cells

Leishmania donovani (AG83) promastigotes were obtained from Indian Institute of Chemical Biology, Kolkata. Cells were cultured in medium M199 (pH 7.4) supplemented with 10% FCS, 100 units·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin at 23 °C as described previously [14], and log-phase cells were harvested in phosphate-buffered (10 mM, pH 7.2) saline (0.15 M).

Cloning and expression of HbR-truncated proteins

Three truncated peptides HbR$^{1–126}$, HbR$^{121–276}$, and HbR$^{270–471}$ corresponding to the N terminus, middle region, and C terminus of LdHbR, respectively, were cloned and expressed as described previously [14]. As maximum Hb-binding activity of HbR retains in HbR$^{1–126}$ with some contribution from HbR$^{121–276}$, we amplified HbR$^{1–260}$ fragment by PCR using appropriate forward (5′-gtggatc catggccacccgcgtgaac-3′) and reverse (5′-gctgagcctatcgctcatc gaattctg-3′) primers from full-length LdHbR as template following identical PCR conditions as described previously [14]. Briefly, PCR was performed with appropriate primers using HbR as template and platinum HiFidelity *Taq* polymerase (Invitrogen) in a thermocycler for 30 cycles (denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 68 °C for 2 min).

To determine minimal Hb-binding domain of HbR, one set of PCRs were carried out using reverse primer (5′-gt gaattcctagccgcgggcagacacgg-3′) along with twelve different forward primers (F1-5′-gtggatccatggccacccgcgtgaac-3′; F2-5′-gtggatcc gagatgcgctacatcaagcag-3′; F3-5′-gtggatcctcctcgga gaagatgaagcag-3′; F4-5′-gtggatccgagggccgcccgagcac-3′; F5-5′-gtggatccaaggccaccggtgtgtactac-3′; F6-5′-gtggatccgtgagcct gcgcggcg-3′; F7-5′-gtggatccagtgccctggttggcgatg-3′; F8-5′-gtg gatccatgatgtcggagaacgccc-3′; F9-5′-gtgg atcctccttcccggtgga ccag-3′; F10-5′-gtggatcc ttctcgacgaagaacgtg-3′; F11-5′-gtg gatcccgcgtgcgcgtcaacgtg-3′; F12-5′-gtggatccgcccgct acttcgtg gac-3′) to generate HbR$^{1–260}$, HbR$^{21–260}$, HbR$^{41–260}$, HbR$^{61–260}$, HbR$^{81–260}$, HbR$^{101–260}$, HbR$^{121–260}$, HbR$^{141–260}$, HbR$^{161–260}$, HbR$^{181–260}$, HbR$^{201–260}$, and HbR$^{221–260}$ deletion mutants that were serially staggered by 20aa from the N terminus of the HbR using similar PCR conditions as described previously.

Similarly, another set of PCRs were carried out using forward primer (5′-gtggatccatggccacccgcgtgaac-3′) along with eight different reverse primers (R8-5′-gtgaattcctagaag gtgaaccccagcgg-3′; R7-5′-gtgaattcctacttcttcacgctctgcgc-3′; R6-5′-gtgaattcctacttcgggatcacgaacttcg-3′; R5-5′-gtgaattcc taacgcaacacgcggaagttc-3′; R4-5′-gtgaattcctaggccgggtcggacgt gtac-3′; R3-5′-gtgaattcctaaagaccctccaccatctcg-3′; R2-5′-gtg aattcctacatggtgaactgggtggcg-3′; R1-5′- gtgaattcctactcgctatcc gagtcg-3′) to generate HbR$^{1–160}$, HbR$^{1–140}$, HbR$^{1–120}$, HbR$^{1–100}$, HbR$^{1–80}$, HbR$^{1–60}$, HbR$^{1–40}$, and HbR$^{1–20}$ deletion mutants staggered by 20 aa from C-terminal end of HbR$^{1–160}$. Finally, all amplified HbR fragments were subcloned into *Bam*HI/*Eco*RI sites of pGEX-4T-2 vector and transformed into *Escherichia coli*. Transformed *E. coli* were grown in LB and induced with isopropyl β-D-thiogalactopyranoside, and recombinant GST-HbR fusion proteins were purified by a standard procedure using reduced glutathione beads. Purified proteins were analyzed by 12% SDS/PAGE as described previously.

Similarly, HbR$^{121–160}$ and HbR$^{361–400}$ were cloned and expressed as GST fusion protein. Briefly, HbR$^{121–160}$ was PCR-amplified using appropriate forward primer (5′-gtg gatccagtgccctggttggcgacg-3′) and reverse primer (5′-gtgaa ttcctagaaggtgaaccccagcggc-3′) and HbR$^{361–400}$ was PCR-amplified using forward primer (5′-gtggatccacccgcgagc

tcatcaagc-3′) and reverse primer (5′-gtgaattcctagaagacagcgc cctgctgagc-3′) from full-length LdHbR as template.

To generate the Hb-binding deficient mutant of $HbR^{41–80}$ ($HbR^{41–80\ mutant}$), $HbR^{41–80}$ encoding nucleotide sequence containing desired mutations, highlighted in bold (5′-tcctcg gagaagatgaagcagctcaccatgtacatgatccacgagatggtg**gcg**ggt**gcagcg** ggc**gcg**ccgagcaccgtg**gcg**atg**gca**ccgtccttcgtgtacacgtccgacccggcc-3′) was synthesized. Using this mutant sequence as template, $HbR^{41–80\ Mutant}$ was PCR-amplified using F3 FP and R4 RP and cloned into pGEX-4T2 as mentioned above. Respective clones were transformed into *E. coli* BL21 competent cells and GST fusion proteins were purified.

Determination of hemoglobin binding with HbR-truncated proteins

Hb binding with different HbR-truncated proteins or peptide was determined using biotinylated hemoglobin (BHb) by a modified enzyme-linked immunosorbent assay as described previously [14]. Briefly, equimolar concentration (200 nM) of HbR-truncated proteins was coated into respective well, washed thrice with PBST (PBS containing 0.1% Tween 20), and incubated for 1 h at 37 °C in blocking buffer (PBST containing 2% gelatin). Wells were washed and incubated with 25 ng of BHb in 100 μL of PBS for 1 h at 37 °C to allow binding. To determine the binding of BHb with respective HbR, wells were washed and incubated with 100 μL (1 $\mu g \cdot mL^{-1}$) of AHRP for 30 min at 37 °C. Finally, unbound AHRP was removed by washing thoroughly with PBST, and the HRP activity present in each well was measured as described [13]. After subtracting the background readings obtained with free GST, which was negligibly low, the HRP activity associated with each truncated HbR protein was expressed as relative binding of hemoglobin with the respective control fragment of HbR, arbitrarily chosen as 100%.

Preparation of axenic amastigotes

To determine the effect of $HbR^{41–80}$ peptide in the Hb uptake and growth of amastigotes, we have prepared axenic amastigotes following similar method as described previously [19]. Briefly, late log-phase promastigotes cells were transferred to M199 medium containing 10% FCS, having pH-5.5 and incubated in CO_2 incubator at 37 °C for 48 h to differentiate into amastigotes. Differentiated amastigotes were grown under same conditions for another 48 h and used for experiments.

Role of HbR peptide in hemoglobin uptake by *Leishmania*

To determine the role of peptide or truncated HbR, Hb uptake in *Leishmania* promastigotes or amastigotes was determined in the presence or absence of peptide or proteins as described previously [17]. Briefly, respective *Leishmania* cells (10^7 cells·mL^{-1}) were washed twice and resuspended in 250 μL of ice-cold vPBS containing Alexa Fluor-594-labeled Hb (100 nM) and incubated for 15 min at 23 °C in the presence or absence of peptide. Cells were washed three times with cold vPBS to remove unbound Alexa-Hb. Finally, cells were resuspended in chilled vPBS and visualized under a Zeiss LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany).

Effect of HbR peptide on the growth of *Leishmania*

To determine whether impairment of Hb uptake by $HbR^{41–80}$ peptide inhibits the growth of promastigote or amastigote forms of *Leishmania*, we have measured the growth of the parasites in the presence or absence of the peptide by [^{3}H]-thymidine incorporation assay as described earlier [17]. Briefly, cells (10^6 cells) were washed, resuspended in 200 μL of sterile M199 medium and were incubated with an equimolar concentration (0.625 nM) of indicated molecules (GST, 40 mer peptide, $HbR^{1–126}$, or $HbR^{270–471}$) containing [^{3}H]-thymidine (0.6 μCi per well) into flat-bottom tissue culture wells for 12 h at 23 °C for promastigotes or at 37 °C for indicated times for amastigotes. Subsequently, cells were harvested and washed with a multiwell cell harvester (Wallac). After harvesting, dry filters were processed to measure the radioactivity incorporated by respective *Leishmania*, using a beta plate liquid scintillation counter. The radioactivity incorporated by the cells was directly proportional to the growth of the parasite.

Statistical analysis

Statistical analysis was performed using SIGMA-PLOT version 12 (Systat Software, San Jose, CA, USA). Student's two-tailed paired *t* test was used to determine differences between control and test groups with 95% confidence intervals. *P* values < 0.05 were considered to be significant for all analyses.

Results and Discussion

Previously, we have demonstrated that *L. donovani* internalizes Hb *via* a high-affinity HbR and this process is essential for the parasite survival [13–17,20]. However, Hb-binding domain of HbR is not yet characterized. In order to identify the Hb-binding region of HbR, three truncated proteins $HbR^{1–126}$, $HbR^{121–276}$, and $HbR^{270–471}$, were cloned and expressed as GST fusion proteins. These truncated proteins were immobilized and their binding with Hb was determined using a modified ELISA as described previously

[14]. Full-length GST-HbR$^{1–471}$ protein was used as control. The results presented in Fig. 1 showed that HbR$^{1–126}$ retains about 80% Hb activity in comparison with HbR$^{1–471}$ protein. In addition, about 30% of Hb binding was observed with HbR$^{121–276}$ in comparison with control protein, whereas significantly less Hb binding (< 20%) was detected with HbR$^{270–461}$ in comparison with full-length HbR. These results indicated that the Hb-binding domain of HbR might exist in HbR$^{1–276}$. These results were found to be consistent with our previous studies that N-terminal of HbR is extracellular Hb-binding domain [14,16].

In order to identify the minimum Hb-binding domain of HbR, the HbR$^{1–260}$ was serially truncated from the N terminus staggered by 20 amino acids. Appropriate primers were used to amplify 12 different truncated fragments of HbR$^{1–260}$ by PCR using full-length HbR as template (Fig. 2A, right panel). These PCR products were subcloned into pGEX-4T-2 vector, expressed, and purified as GST fusion proteins. The purified truncated proteins, namely HbR$^{1–260}$, HbR$^{21–260}$, HbR$^{41–260}$, HbR$^{61–260}$, HbR$^{81–260}$, HbR$^{101–260}$, HbR$^{121–260}$, HbR$^{141–260}$, HbR$^{161–260}$, HbR$^{181–260}$, HbR$^{201–260}$, and HbR$^{221–260}$, were analyzed by SDS/PAGE and found to be of expected size (Fig. 2A, left panel). Subsequently, Hb-binding activities of these truncated proteins were analyzed using similar assay as described previously. The results presented in Fig. 2B showed that HbR$^{1–260}$, HbR$^{21–260}$, HbR$^{41–260}$, and HbR$^{61–260}$ retained about 90% Hb-binding activity whereas about 50% inhibition of Hb binding was observed with HbR$^{81–260}$, HbR$^{101–260}$, or HbR$^{121–260}$ in comparison with HbR$^{1–260}$. However, about 70% loss in Hb-binding activity was observed with HbR$^{141–260}$ in comparison with control protein. No significant Hb-binding activities were detected with HbR$^{161–260}$, HbR$^{181–260}$, HbR$^{201–260}$, and HbR$^{221–260}$ truncated proteins. These results suggested that full Hb-binding activity of HbR is possibly located within first 80 amino acid residues of the N terminus of the protein.

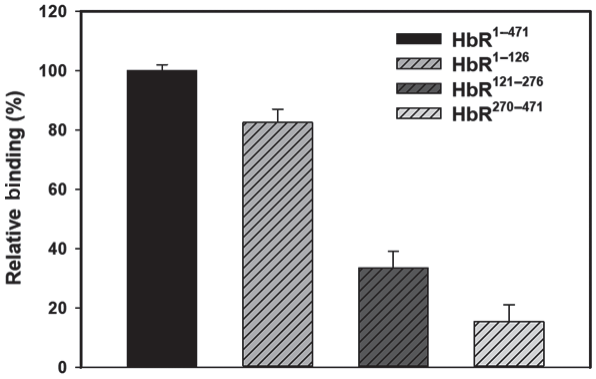


Fig. 1. Identification of the hemoglobin-binding domain of HbR. The deletion mutants HbR$^{1–126}$, HbR$^{121–276}$, and HbR$^{270–471}$ were cloned and expressed as GST fusion proteins, and Hb binding was detected using biotinylated-Hb as described in Materials and methods. The Hb-binding activity detected with HbR$^{1–471}$ was taken as 100% and results are expressed as an average of relative Hb binding of three independent experiments ± SE.

To narrow down the residues in the N-terminal end of the HbR involved in Hb binding, HbR$^{1–160}$ was serially truncated from the C terminus staggered by 20 amino acids. Appropriate primers were used to amplify eight different truncated fragments of HbR$^{1–160}$ by PCR using full-length HbR as template (Fig. 2C, right panel). These PCR products were subcloned into pGEX-4T-2 vector, expressed, and purified as GST fusion proteins. The purified truncated proteins, namely HbR$^{1–160}$, HbR$^{1–140}$, HbR$^{1–120}$, HbR$^{1–100}$, HbR$^{1–80}$, HbR$^{1–60}$, HbR$^{1–40}$, and HbR$^{1–20}$, were analyzed by SDS/PAGE and found to be of expected size (Fig. 2C, left panel). Subsequently, truncated proteins were analyzed for their Hb-binding activity. In correlation with previous finding, our results showed that more than 90% of the Hb-binding activity is retained with HbR$^{1–160}$, HbR$^{1–140}$, HbR$^{1–120}$, HbR$^{1–100}$, and HbR$^{1–80}$ truncated proteins in comparison with HbR$^{1–260}$ (Fig. 2D). However, about 30% loss in Hb-binding activity was observed with HbR$^{1–60}$. No significant Hb-binding activity was detected with HbR$^{1–40}$ and HbR$^{1–20}$ in comparison with control protein indicating that first 40 amino acid residues from the N terminus of HbR do not contribute to Hb binding. Though residues span between HbR$^{81–260}$ also showed partial Hb-binding activity, maximum Hb-binding activity was found to be within HbR$^{1–80}$. Cumulative analysis of N terminus and C terminus truncations of HbR indicated that HbR$^{41–80}$ is predominantly Hb-binding domain of HbR (Fig. 2E). Sequence analysis of HbR$^{41–80}$ revealed that putative Hb-binding domain of HbR consists of SSEKMKQLTMYMIHEMVEGLEGRPSTVR MLPS FVYTSDPA amino acids.

To confirm HbR$^{41–80}$ as Hb-binding domain of HbR, HbR$^{41–80}$ truncated protein was cloned and expressed as GST fusion protein and its Hb binding was determined. The results presented in Fig. 3A showed that more than 90% of Hb binding is retained in HbR$^{41–80}$ in comparison with full-length HbR$^{1–471}$ and HbR$^{1–126}$ proteins. As *Leishmania* acquires heme from Hb endocytosis [13], therefore, we determined whether GST-HbR$^{41–80}$ specifically inhibits Hb endocytosis in parasite. Accordingly, cells were incubated with Alexa Fluor-594-labeled Hb (100 nM) for 15 min

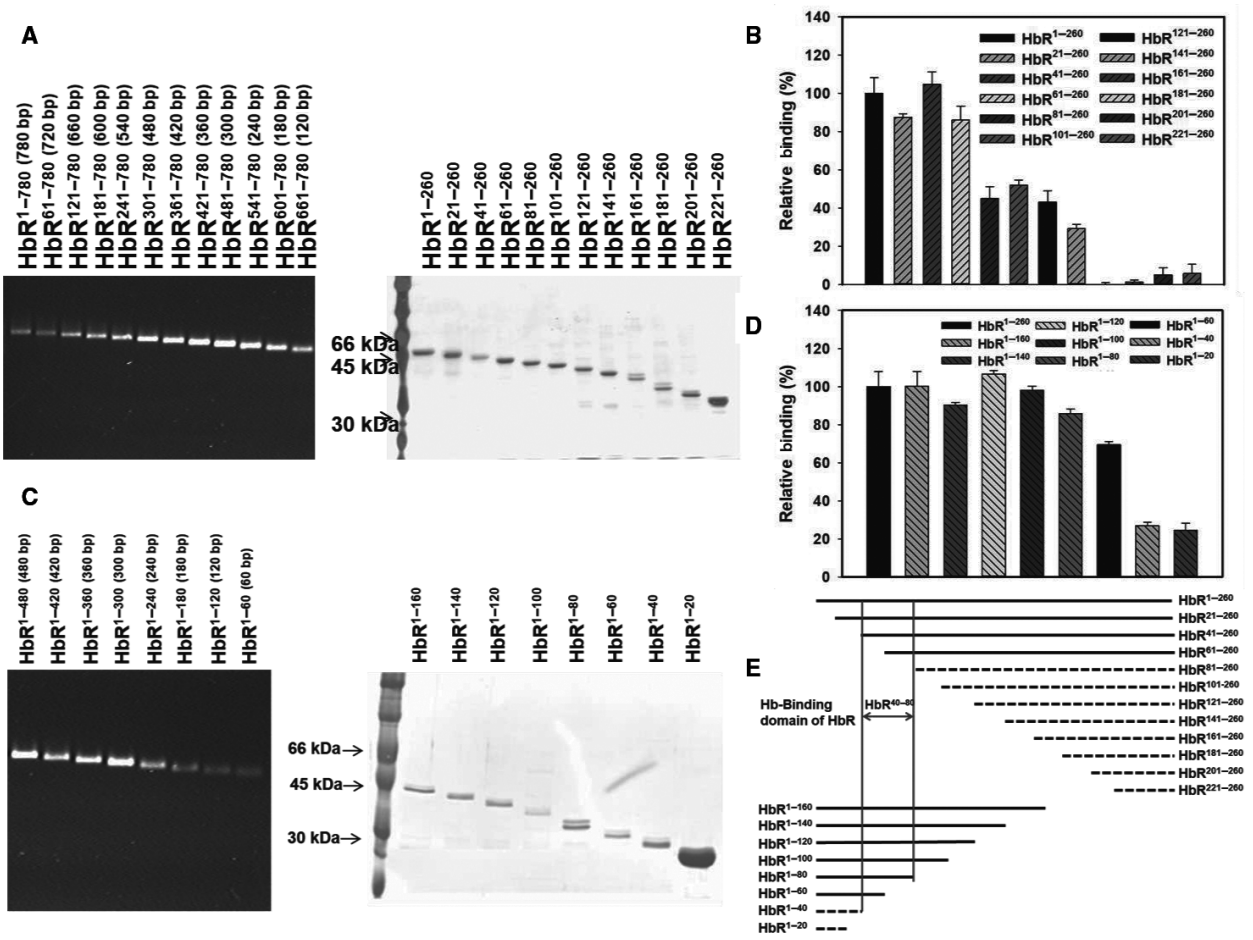


Fig. 2. Identification of the minimum hemoglobin-binding domain of HbR. (A) PCR amplification (right panel) and SDS/PAGE analysis (left panel) of 12 truncated mutants of HbR from the N terminus of $HbR^{1–260}$ as described in Materials and methods. All results are representative of three independent experiments. (B) Equimolar amount of indicated HbR-truncated mutants was immobilized into respective wells, and Hb binding was detected as described in Materials and methods. The Hb-binding activity detected with $HbR^{1–260}$ was taken as 100% and results are expressed as an average of relative Hb binding of three independent experiments ± SE. (C) PCR amplification (right panel) and SDS/PAGE analysis (left panel) of eight truncated mutants of HbR from the C terminus of $HbR^{1–160}$ as described in Materials and methods. All results are representative of three independent experiments. (D) Equimolar amount of indicated HbR-truncated mutants were immobilized into respective wells and Hb binding was detected using biotinylated-Hb as described in Materials and methods. The Hb-binding activity detected with $HbR^{1–260}$ was taken as 100% and results are expressed as an average of relative Hb binding of three independent experiments ± SE. (E) Diagrammatic representation of HbR-truncated mutants serially staggered by 20 aa from the N terminus of $HbR^{1–260}$ (upper panel) or serially staggered by 20 aa from C-terminal end of $HbR^{1–160}$ (lower panel). All mutants which retain significant Hb-binding activity are depicted by solid line while mutants showing diminished Hb binding are indicated by dotted line. The predicted Hb-binding region of HbR appears to be spanning between 40–80 aa from N-terminal of HbR as indicated by arrow.

at 23 °C in the presence of 100-folds (10 μM) excess of indicated proteins. Our results showed that $HbR^{41–80}$ completely blocked Hb endocytosis in parasites. No significant change in Hb endocytosis was observed in the presence of $HbR^{121–160}$ or $HbR^{361–400}$ (Fig. 3B). These results confirmed that Hb-binding domain of HbR resides within $HbR^{41–80}$.

To unequivocally prove the role of identified 40 amino acid residues in Hb binding, a peptide was synthesized (SSEKMKQLTMYMIHEMVEGLEGRPS TVRML PSFVYTSDPA) from a commercial facility and Hb-binding activity was determined. Briefly, peptide (19.38 μM) was immobilized into the well and Hb binding with peptide was determined using indicated concentrations of biotinylated-Hb as described previously. Interestingly, our results showed that peptide binds with Hb and maximum binding of immobilized peptide is obtained at 10 ng biotinylated-Hb (Fig. 4A).

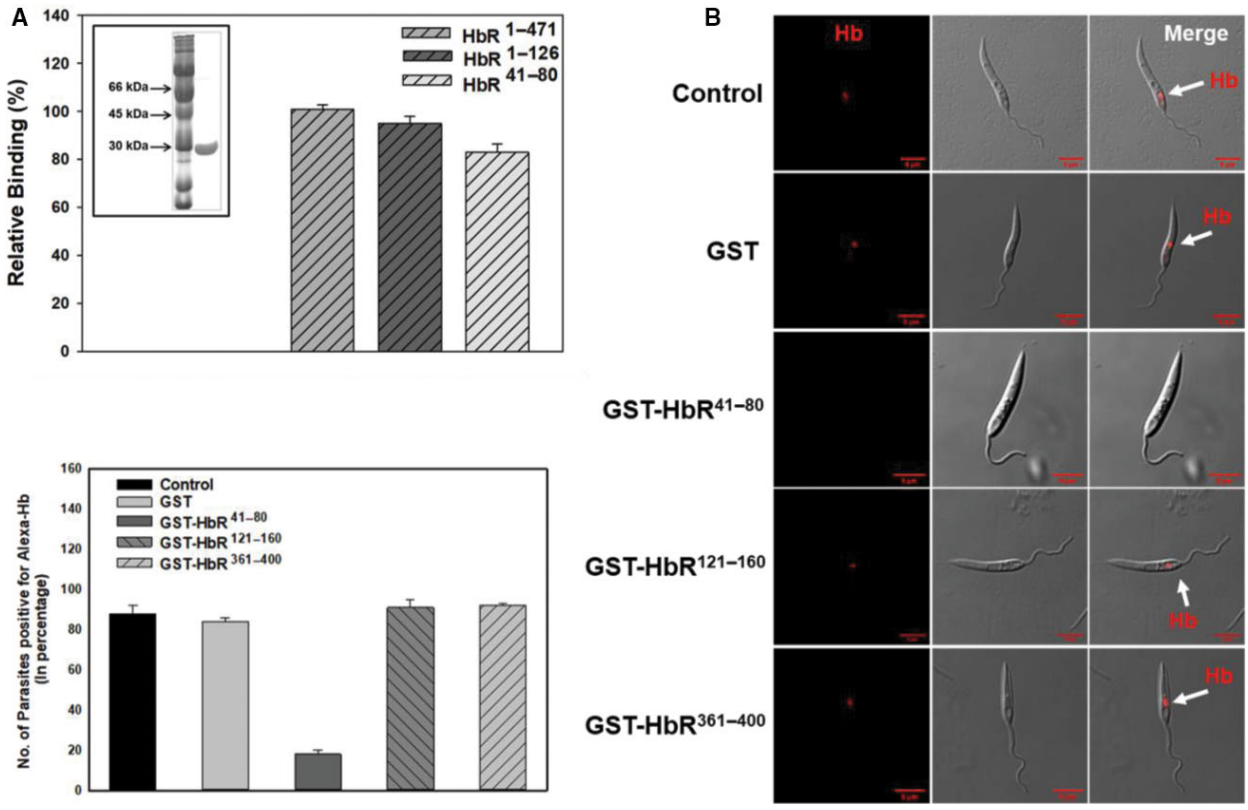
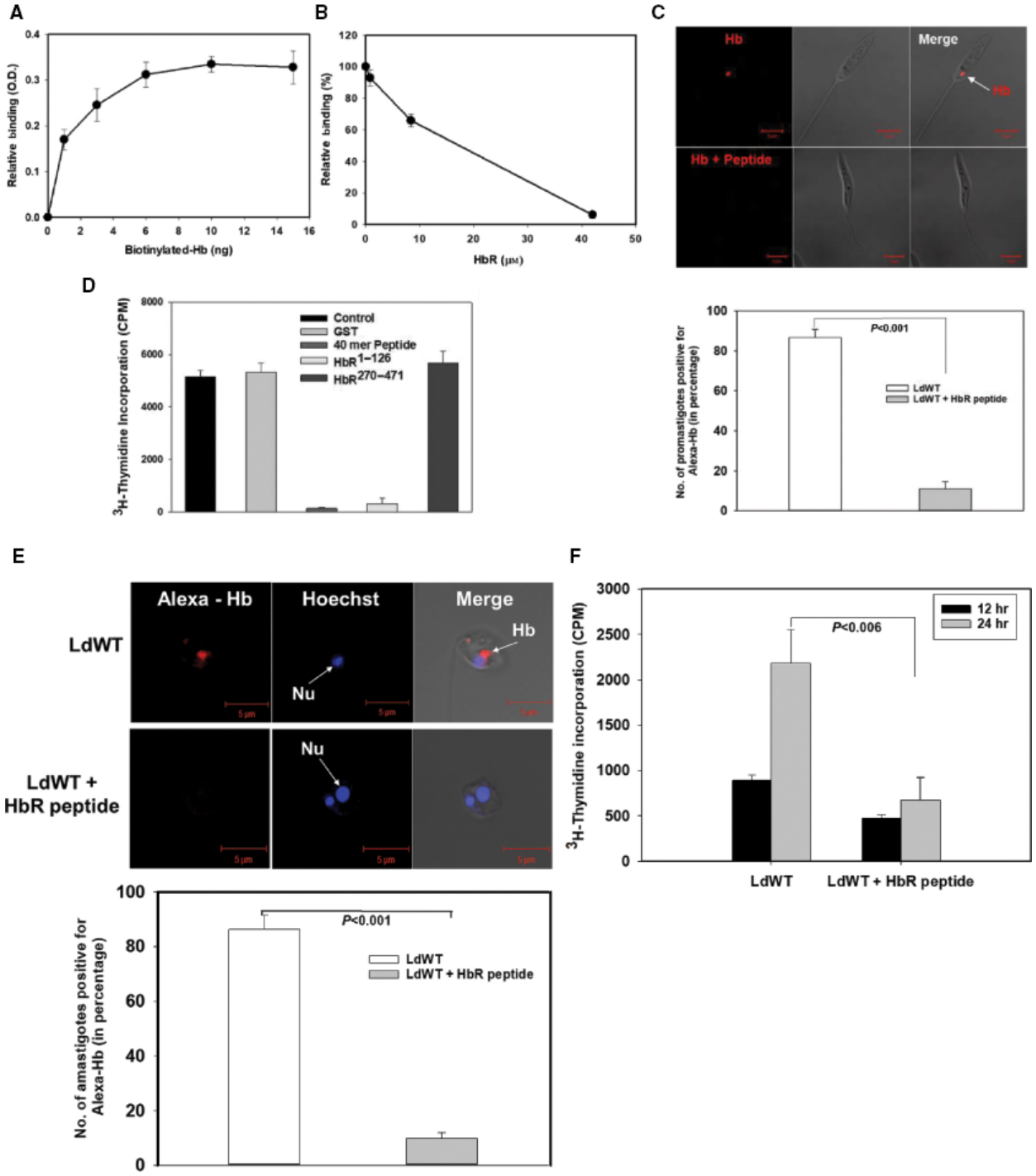


Fig. 3. Determination of $HbR^{41–80}$ as Hb-binding domain of HbR. (A) To determine $HbR^{41–80}$ as Hb-binding domain of HbR, indicated purified proteins were immobilized and Hb binding was determined as described in Materials and methods. The Hb-binding activity detected with $HbR^{1–471}$ was taken as 100% and results are expressed as an average of relative Hb binding of three independent experiments ± SE. Inset shows the purified $HbR^{41–80}$ as GST fusion protein. (B) To determine whether $HbR^{41–80}$ specifically inhibit Hb endocytosis in *Leishmania*, $HbR^{41–80}$, $HbR^{121–160}$, and $HbR^{361–400}$ were cloned and expressed as GST fusion proteins. *Leishmania* promastigotes were incubated with Alexa-594-Hb for 15 min at 23 °C in the presence or absence of equimolar concentration of indicated proteins. Cells were washed, fixed, and visualized under confocal microscope to determine the Hb uptake as described in Materials and methods. Results are representative of three independent experiments. Left panel shows the quantitation of the results from at least 100 observations.

In order to determine the specificity, 38.76 μM peptide was immobilized and Hb binding with peptide was measured in the presence of indicated concentrations of HbR. Almost 100% Hb binding with peptide was inhibited in the presence of 42 μM of HbR demonstrating that synthesized peptide specifically binds with Hb (Fig. 4B). Subsequently, we determined whether synthesized peptide inhibits Hb endocytosis in parasite. Accordingly, cells were incubated with Alexa Fluor-594-labeled Hb (100 nM) for 15 min at 23 °C in the presence of 100-folds (10 μM) excess of peptide. Our results showed that Hb endocytosis was completely inhibited by the peptide (Fig. 4C). Previously, we showed that blocking of hemoglobin endocytosis is detrimental for the growth of the parasites [13–17,20]; therefore, we determined the growth of the parasites in the presence of peptide. Our results showed that addition of equimolar concentration (0.625 nM) of peptide or $HbR^{1–126}$ inhibits more than 95% growth of the parasites in comparison with control (Fig. 4D).

Recently, we demonstrated that intracellular amastigotes also express Hb receptor and mediates Hb endocytosis through this receptor to acquire heme from the degradation of internalized Hb for their intracellular survival [21]. Therefore, we checked whether $HbR^{41–80}$ peptide inhibits Hb uptake in amastigotes. Our results showed that $HbR^{41–80}$ peptide significantly blocked the Hb endocytosis in amastigotes (Fig. 4E). Further analysis of at least 100 amastigotes revealed that more than 90% of amstigotes failed to mediate Hb internalization in the presence of $HbR^{41–80}$ peptide in comparison with untreated control. Consequently, we found that addition of HbR peptide inhibits more than 80% growth of the amastigotes in comparison with untreated control (Fig. 4F). Taken together, these results demonstrated that synthesized



peptide inhibits Hb endocytosis and thereby growth of both *Leishmania* promatigotes and amastigotes. Previously, our results showed that HbR is well conserved in various species of *Leishmania*, for example, *L. amazonensis, L. infantum, L. donovani, L. major, L. turanica, L. gerbilli, L. tropica*, and *L. enrietti* with 97–100% sequence identity [18]. Thus, it is tempting to speculate that HbR$^{41–80}$ peptide may inhibit the growth of other species of *Leishmania*.

To determine critical residues of HbR$^{41–80}$ involved in the Hb binding, we modeled the structure of the full-length HbR based on homology with crystal

Fig. 4. Characterization of the 40-mer HbR peptide in Hb binding and uptake in *Leishmania*. (A) To unequivocally prove the role of identified 40 amino acid residues in Hb binding, synthesized peptide (19.38 μM) was immobilized into the well and binding of indicated concentration of Hb was determined as described in Materials and methods. Results are expressed as an average of three independent experiments ± SE. (B) To determine the specificity, 38.76 μM peptide was immobilized and Hb binding with peptide was measured in the presence of indicated concentrations of HbR. Hb binding with peptide in the absence of competing HbR was taken as 100% and results are expressed as an average of relative Hb binding of three independent experiments ± SE. (C) To determine whether 40-mer peptide inhibit Hb uptake in *Leishmania*, respective *Leishmania* promastigotes were incubated in the presence or absence of peptide for 15 min at 23 °C and Hb uptake by the cells were determined as described in Materials and methods. Results are representative of three independent experiments. (D) To determine the effect of $HbR^{41–80}$ peptide on the growth of *Leishmania* promastigotes, growth of the parasites in the in the presence of equimolar concentration (0.625 nM) of GST, 40-mer peptide, $HbR^{1–126}$, or $HbR^{270–471}$ measured as Materials and methods. Results are expressed as mean ± SD of three independent experiments. (E) To determine whether 40-mer HbR peptide also blocks the Hb uptake in amastigotes, cells were incubated in the presence or absence of peptide for 15 min at 23 °C and Hb uptake by the cells were determined as described for promastigotes in Materials and methods. Results are representative of three independent experiments. Lower panel shows the quantitation of the results from at least 100 observations. (F) To determine the effect of $HbR^{41–80}$ peptide on the growth of *Leishmania* amastigotes, cells were incubated in the presence or absence of peptide for different period of times and growth of the amastigotes was measured as described for promastigotes in Materials and methods. Results are expressed as mean ± SD of three independent experiments.

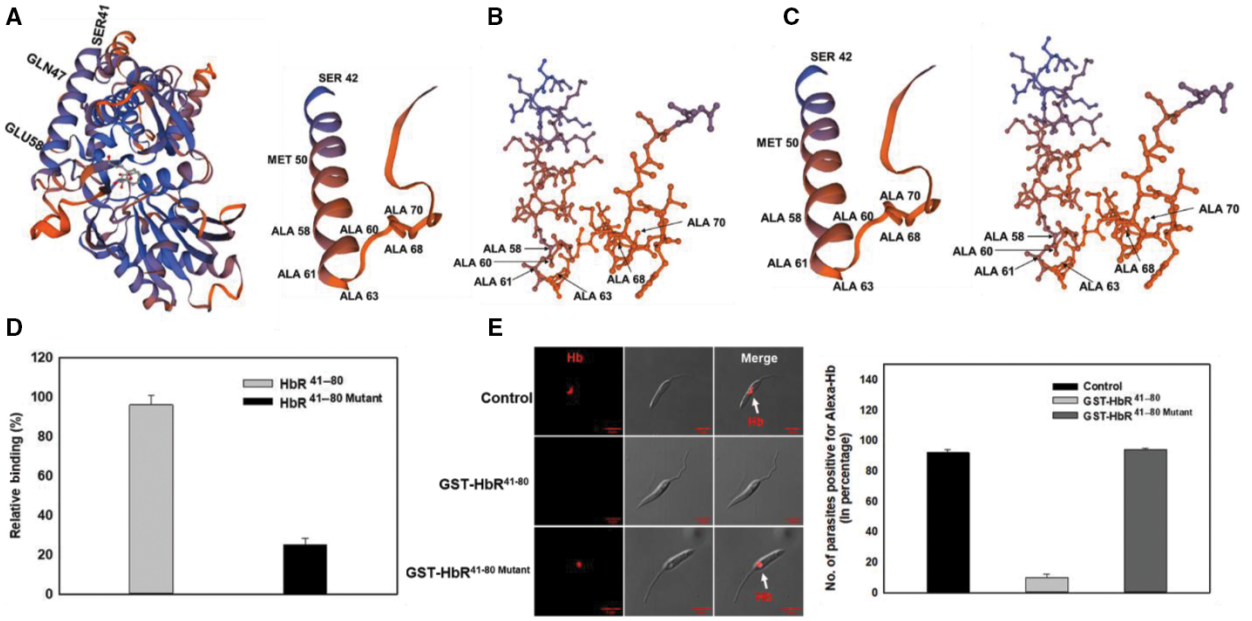


Fig. 5. Generation and evaluation of $HbR^{41–80}$ mutant in Hb binding and uptake in *Leishmania*. (A) Homology-based stuructural model of *Leishmania* full-length HbR by swissmodel showing 41–60 residues of HbR form a helix, HbR 61–70 forms a turn while HbR 70–80 has an extended conformation and the segment of 41–70 residues of HbR is present on surface of HbR. (B) The structural model of $HbR^{41–80}$ also showing very similar conformation and charged residues Glu 58, Arg 63, and Arg 68 exposed to solvent on the surface of HbR. (C) The structural model of alanine-substitution $HbR^{41–80\ mutant}$ showing the rearrangements in backbone conformation and loss of solvent-exposed charged side chains of indicated residues. (D) To determine Hb-binding activity of $HbR^{41–80}$ and $HbR^{41–80\ mutant}$, indicated proteins were purified as GST fusion proteins and Hb binding was determined as described in Materials and methods. The Hb-binding activity detected with $HbR^{41–80}$ was taken as 100% and results are expressed as an average of relative Hb binding of three independent experiments ± S.E. (E) To determine the role of $HbR^{41–80\ mutant}$ in Hb endocytosis in *Leishmania* promastigotes, cells were incubated with Alexa-594-Hb for 15 min at 23 °C in the presence of equimolar concentration of $GST\text{-}HbR^{41–80}$ or $GST\text{-}HbR^{41–80\ mutant}$ and Hb uptake was determined as described in Materials and methods. Results are representative of three independent experiments. Right panel shows the quantitation of the results from at least 100 observations.

structure of recombinant human Hexokinase type I using swissmodel software. Homology-based structural model of *Leishmania* HbR indicated that 41–60 residues of HbR forms a helix and HbR 61–70 forms a turn while HbR 70–80 has an extended conformation. Moreover, modeled structure of HbR also

showed that the segment of 41–70 residues of HbR is present on surface of HbR (Fig. 5A). Thus, structural model of HbR also supported our finding that this region of HbR can be potentially involved in the binding with Hb. In addition, the structural model (Swiss model) of $HbR^{41–80}$ also showed very similar conformation like the indicated segment in HbR with charged residues Glu 58, Arg 63, and Arg 68 exposed to solvent on the surface of HbR (Fig. 5B). Therefore, series of alanine-substitution mutants [22] were made (E58A, L60A, E61A, R63A, R68A, L70A) to prepare a $HBR^{41–80\ mutant}$ (SSEKMKQLTMYMI HEMVAGAAGAPSTVAMAPSFVYTSDPA) and Hb binding was determined. Homology-based model of the $HbR^{41–80\ mutant}$ structure showed the rearrangements in backbone conformation in the segment 58–70 of HbR and loss of solvent-exposed charged side chains of E58, E61, R63, and R68 (Fig. 5C). These results suggested that there could be potential loss of Hb binding of $HbR^{41–80\ mutant}$. Indeed, the results presented in Fig. 5D showed about 80% loss of Hb-binding ability of GST-$HbR^{41–80\ mutant}$ in comparison with $HbR^{41–80}$ (Fig. 5D). Consequently, we found that GST-$HbR^{41–80\ mutant}$ fails to inhibit the Hb endocytosis in *Leishmania* promastigotes in comparison with untreated control cells (Fig. 5E), whereas GST-$HbR^{41–80}$ completly blocked the Hb endocytosis under identical conditions. Apart from *Leishmania*, other pathogenic protozoan parasites like *Plasmodium* and *Trypanosoma* also endocytosed Hb from the host cells. Previous studies showed that malaria parasite internalizes hemoglobin *via* a specialized structure called 'Big Gulp' from red blood cells [23]; however, no specific receptor for Hb in *Plasmodium* is reported yet. Whereas, it was demonstrated [24] that Hb uptake in *Trypanosoma* is mediated through haptoglobin–hemoglobin receptor (HpHbR). Structural analysis of TbHpHbR revealed that HpHbR is an elongated three α-helical bundle with a small head structure. The head consisted of a 42-residue-long loop which was shown to be ligand-binding site by series of alanine-substitution mutants [22]. However, hexokinase as a receptor for Hb is not yet reported. But, cell surface-associated glyceraldehyde-3-phosphate dehydrogenase was shown to be involved in iron acquisition in *Staphylococcus* [25]. Similarly, a 95-kDa phosphotyrosine-containing protein on the surface of mouse spermatozoa was reported to be a receptor for ZP3 [26] which was subsequently identified as hexokinase [27].

Interestingly, previous studies showed that peptide blocking protein–protein interaction by mimicking one of their binding domain is attractive candidate for drug design against various diseases [28,29]. In correlation with our studies, recent studies showed that about 7000 peptides are identified predominantly against extracellular targets and cell surface receptors; and large numbers of these mimicking peptides are approved for clinical trials [30]. In addition, antimicrobial peptides were also used as anti-Leishmanial agent [31,32]. For example, peptides designed from *Leishmania* GP63 inhibited growth of *L. major in vitro* and reduced cutaneous lesions in BALB/c mice [33]. Thus, peptide corresponding Hb-binding domain of *Leishmania* species might be useful as an anti-leishmanial agent in different forms of leishmaniasis.

In conclusion, this is the first demonstration of the identification of 40 amino acid residues as Hb-binding domain of HbR. Subsequently, our results have shown that a peptide corresponding to the Hb-binding domain of HbR inhibits Hb endocytosis in *Leishmania* and, thereby, prevents the growth of the parasites. This finding is very important as HbR is well conserved in all different species of *Leishmania* and the parasite acquires heme from internalized Hb *via* this receptor. Thus, the identified peptide has potential in the development of novel therapeutic agents against Leishmaniasis, which will be explored in the future.

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Author contributions

AM conceived and coordinated the study and wrote the paper. RR, GK, VS and CS, IA, JKV, AK performed experiments. JKV, AK, and KK designed experiments and analyzed results.

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