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Caveolin-1 binding motif of α-hemolysin: its role in stability and pore formation

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Abstract

We have identified a nine amino sequence in α-hemolysin (α-HL) of *Staphylococcus aureus*, which binds Caveolin-1. Surface plasmon resonance studies clearly show a concentration dependent interaction of α-HL with the scaffolding domain of Caveolin-1. Mutants of α-HL, devoid of Caveolin-1 recognition motif, exhibit an α-HL like proteinase K digestion profile but the resultant 'half-like' domains are highly susceptible to further proteolysis. They also had the same intrinsic fluorescence emission maxima as the native α-HL indicating normal folding. However, these mutants bind 1-anilino-8-naphthalene sulfonic acid probably due to exposure of their hydrophobic core. Moreover, these mutants are non-lytic and do not undergo conformational changes on rabbit RBC membrane surface. Purified Caveolin-1 blocks the hemolysis of RBCs by α-HL. Our studies indicate that the Caveolin-1 binding motif of α-HL provides stability and shields the hydrophobic core of α-HL. The motif also acts as trigger point for initiation of conformational changes.

Keywords: α-Hemolysin; Caveolin-1 binding motif; Scaffolding domain; Hetero-oligomerization; Fluorescence studies; ANS binding

Transmembrane β-barrel structure is commonly encountered in bacterial outer membrane proteins and pore-forming toxins such as α-hemolysin (α-HL) of *Staphylococcus aureus* [1–6]. α-HL is a pore-forming, cytotoxin, that assembles into a heptameric β-barrel from a water-soluble monomer on the surface of susceptible cells, perforating the cell membranes, thereby leading to death [1]. Although the heptameric crystal structure of α-HL which exhibits the β-barrel is known, the structure of water-soluble monomeric form is yet to be delineated [2]. Recently, the crystal structure of an α-HL heptamer bound to phosphatidylcholine was reported by Gouaux and co-worker [7]. The studies on the interactions between α-HL and PC head groups have provided an insight into the nature of interactions that α-HL evokes for its assembly in artificial membranes [8–11]. Although PC head groups are present in several natural cell membranes, the α-HL did not penetrate the human granulocyte cell membranes [12]. This gives rise to the possibility of the existence of specific receptors and/or certain structural components on mammalian cell surface, that are exploited by α-HL for its penetration on susceptible cells. Studies on the assembly of α-HL employing the artificial membranes have concluded that the α-HL does not damage large liposomes [13,14] and the pH of the exterior (bulk as well as local) has to be lower than 6 for leakage of liposome encapsulated marker agents [15]. In addition, coexistence of unsaturated fatty acid chains is a pre-requisite for the assembly of α-HL on artificial membranes [9,13,14]. Hence, studies employing artificial membranes as an in vitro experimental paradigm for understanding the α-HL assembly do not define or explain the sensitivity and resistance of α-HL attack on certain mammalian cells, since the same PC or PC like head groups are found in a variety of

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mammalian cells. In addition, it has been felt that the pore size observed on natural cell membranes is smaller than that of the size observed on artificial bilayers. For example, 'small,' but not 'large,' pores were found to be responsible for inducing apoptosis in T-cells [16]. All these observations indicate that the α-HL might exploit some unknown molecules that are embedded in the membrane for its pore formation. In this communication, we report a nine amino acid sequence in α-HL which binds to the Caveolin-1 scaffolding domain, found in most mammalian cells. We have provided several lines of evidences, which indicate that: (i) Caveolin-1 blocks the hemolysis of rabbit RBCs by α-HL, (ii) identified sequence provides stability to water-soluble form of α-HL, (iii) shields the hydrophobic core of α-HL, and (iv) is probably essential for initiation of conformational changes leading to pore formation. In the absence of this nine amino acid sequence, the mutants do not undergo any conformational changes, supporting the concept that the conformational changes in α-HL are probably triggered by specific interaction of this α-HL sequence with target membrane components.

Materials and methods

The Ph.D.-12 Phage Display Peptide Library kit was obtained from New England Biolabs. 1-Anilino-8-naphthalene sulfonic acid (ANS) was purchased from Sigma Chemical, USA. Biotinylated Cav-1 peptide DGIWASFTTFTVTKYEFYR (scaffolding domain peptide described in Couet et al. [17]), and LP (QNWGPYDRDSWNPVY) peptides were obtained by chemical synthesis from Geno-Mechanix, Florida USA. Protein estimations were carried out by Bradford protein estimation kit from Bio-Rad, USA. All other chemicals were of the highest purity available.

HPLC purification of Cav-1, LP peptides. Peptides employed in this study were purified by Symmetry C-18 column by reverse-phase HPLC. The gradient is 10–70% acetonitrile in 60 min (0.1% TFA in water as buffer A and 0.1% TFA in acetonitrile as Buffer B). The molecular weight and biotinylation were confirmed by mass spectroscopy.

Purification of α-HL. Native as well as recombinant α-HL was purified as described earlier [18] and its purity was routinely examined by SDS–PAGE according to the method of Laemmli [19].

Purification of recombinant Caveolin-1. The Caveolin-1 gene (1–101 amino acids) was cloned in pET28-a (+) vector with an amino terminal six histidine tag. The recombinant Caveolin-1 was expressed in *E. coli* and purified with the help of Ni–NTA resin chromatography. The purity was adjudged by SDS–PAGE and the overall preparation by transmission electron microscopy by negative staining.

Cloning, expression, and purification of α-HL-SD and α-HL-LD deletion mutants. α-HL is a 293 amino acid long protein (A^1DSDI...EEMTN^{293}) and the α-HL-SD (small deletion) mutant is made by deleting nine amino acids, i.e., 179–187 (**W^{179}-G-P-Y-D-R-D-S-W^{187}**). α-HL-LD (large deletion) mutant is devoid of 35 amino acids, i.e., 179–213 (**W^{179}-G-P-Y-D-R-D-S-W**-N-P-V^{190}-Y-G-N-Q-L-F-M-K-T-R^{200}-N-G-S-M-K-A-A-D-N-F^{210}-L-D-P^{213}). The α-HL-SD and α-HL-LD were generated by joining PCR products 1 (1–178 amino acids) and 2 (188–293 amino acids) and 1 (1–178 amino acids) and 3 (214–293 amino acids) with the help of a unique *Bam*HI site (Gly-Ser).

Ten milliliter cultures of LB containing 100 μg/ml ampicillin were initiated from a single colony of freshly transformed plates and grown overnight at 37 °C overnight. One liter cultures were inoculated the following morning and were allowed to grow for 3 h. Protein expression was induced with 1 mM IPTG, and cultures were allowed to grow for an additional 6 h. The bacteria were pelleted in 250 ml bottles, at 3000*g* for 15 min at 4 °C. It was found that most of the protein existed in the form of inclusion bodies. The cells were resuspended in 15 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA and 1 M urea) and kept at RT for 1 h. The cells were then sonicated 20× for 30 s. The insoluble protein was recovered by centrifugation at 12,000*g* for 15 min at 4 °C. Inclusion bodies were washed with 50 mM Tris–HCl, pH 8.0, containing 0.1% Triton X-100, 5 mM EDTA, and 1 M urea until maximum amounts of impurity are removed. Two additional washes were carried out with 50 mM Tris–HCl, pH 8.0. The inclusion bodies were solubilized with 8 M urea in 50 mM phosphate buffer and refolding was done by dilution with same buffer without urea. The purity and activity of α-HL, α-HL-SD, and α-HL-LD were routinely examined by SDS–PAGE and hemolysis assays.

Generation of [^{35}S]methionine labeled proteins. [^{35}S]Methionine labeling of toxins was achieved by in vitro transcription and translation using *Escherichia coli* T7-S30 extract system for circular DNA (L-1130, Promega, USA) as described earlier [18]. Briefly, the composition of the mix for one reaction is as follows: supercoiled plasmid (500 ng), S30 premix, (20 μl), T7-S30 extract (15 μl), amino acid mixture minus methionine (5 μl), RNasin H (25 U), rifampicin (50 ng), [^{35}S]methionine 1 μl of 15 μCi/μl), and nuclease free water to 50 μl are mixed and incubated at 37 °C for 90 min. At the end of the incubation, the samples were centrifuged at 16,000*g* for 5 min and the supernatant was immediately used for the experiment.

Bacteriophage display. The Ph.D.-12 Phage Display Peptide Library kit contains a random peptide 12-mer phage display library. *E. coli* ER2738 was used as a host strain for phage amplification. Phage display studies were essentially carried out as described in the manual provided with the kit. Briefly, each round was started by coating the 96-well plates with biotinylated-LP peptide of α-HL or α-HL at 100 ng/well overnight with mild shaking in coating buffer (0.1 M $NaHCO_3$, pH 8.0) at 4 °C. After discarding the coating buffer containing α-HL or LP peptide, the wells were blocked with BSA for 1 h at 4 °C. An aliquot of original library (5 μl) was used in 1st round. In subsequent rounds, the input was 100 μl of amplified elute from previous round. Wells were then washed 6 times with TBST to remove weakly bound phages. Bound phages were eluted by adding the coating protein/LP peptide at a concentration of 100 μg/ml. The phage elute was used to infect the *E. coli* ER2738 at mid-log-phase (OD = 0.5). After 6 h of culture, phages were purified from the culture supernatant using PEG precipitation. Phage elute from the third round of affinity purification was used to infect *E. coli* ER2738 and plated on LB plates with isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactoside to obtain single plaques. Sequencing templates (ssDNA) were prepared from individual plaques to obtain the nucleotide sequence of the peptide representing the phage. The interaction between α-hemolysin and selected phage was further studied by ELISA.

Limited proteolysis on α-HL loop altered mutants of short and long deletion of loop. The purified protein (16 μg) was incubated with 250 ng proteinase K at room temperature. At indicated times, the digestion was stopped by the addition of 1× LSB containing phenylmethylsulfonyl fluoride and heating the sample at 95 °C for 5 min. The samples were then analyzed by 15% of SDS–PAGE, followed by Coomassie blue staining. The band patterns of loop deletion mutants were compared with the previously reported pattern for recombinant α-HL after a similar digestion [18].

Circular-dichroism studies. Far UV-CD (200–250) and Near UV-CD (250–300) spectra of α-HL-SD and α-HL-LD (1 mg/ml) were recorded on a Jasco J715 spectropolarimeter in a 1 mm path length cell equipped with a circulating water bath as reported earlier [18]. The spectra were collected with a response time of 4 s and a scan speed of 100 nm s^{-1}. Each data point was an average of 10 accumulations.

Intrinsic fluorescence emission measurements of α-HL, α-HL-SD, and α-HL-LD. Experiment was performed by using Perkin–Elmer LS-50B spectrofluorometer with spectral band width 5 nm for both excitation and emission. All spectra were corrected with appropriate buffer. Fluorescence measurements were performed after dilution of stock sample to 30 μg/ml with 10 mM Mops, pH 7.0. IFE of α-HL, α-HL- SD, and α-HL-LD were measured with excitation wavelengths at both 280 and 295 nm.

ANS binding experiments. The binding of ANS to recombinant protein or mutant protein was monitored by ANS fluorescence intensity and the shift of λ_{max}. Protein at 30 μg/ml was incubated with different amounts of ANS having a stock concentration of 5 mM. The ANS fluorescence emission spectra were recorded on spectrofluorimeter. Both excitation and emission bandwidths were 5 nm. The protein and ANS mixture was incubated in 10 mM Mops, pH 7.0, for 15 min. The incubated solution was then excited at 380 nm and the fluorescence changes were subsequently monitored between 400 and 600 nm at 25 °C.

Hetero-oligomerization assay. α-HL (30 ng/ml) was mixed with increasing amounts of α-HL-SD or α-HL-LD in K-PBSA and rRBC (500 μl of 2%) was added to a final volume of 1 ml and incubated at 37 °C for 30 min. The amount of cell lysis (hemoglobin release) was measured calorimetrically at 595 nm. One hundred percent lysis was estimated by lysing the red blood cells in 5 mM phosphatase buffer and the percent lysis was calculated.

Results and discussion

The molecule(s) that aid α-HL in its assembly on nucleated cells have not yet been fully identified. Since α-HL can form pores on artificial membranes, especially the PC containing phospholipids, it is generally assumed that α-HL may not have specific receptors in mammalian cells. Considering the requirements of α-HL's assembly in artificial membranes, such as coexistence of unsaturated fatty acids, pH, and size of the vesicles, it appears that the α-HL might assemble with the help of certain structural components of the biological membranes, since, not all cells are susceptible to α-HL attack. We have scanned for motifs in α-HL that have the potential to interact with known biological membrane proteins, whose binding motifs are well elucidated. Based on such an approach, we found that the α-HL contains a motif, viz. **W**^{179}-G-P-**Y**-D-R-D-S-**W**^{187}, which resembles the Caveolin-1 recognition motif. The Caveolin-1 binding motifs, reported in the literature, are ΦXΦ XXXXΦ or ΦXXXXΦXXΦ or the combined motif ΦX-ΦXXXXΦXXΦ where Φ is any aromatic amino acid (W, F or Y) [20–22]. However, the α-HL contains an extra amino acid, i.e., P^{181}. In view of this resemblance, the role of this motif in the interaction of α-HL with Caveolin-1 and its role in folding and function of α-HL have been studied.

Screening of α-HL interacting peptides

The Ph.D.-12 phage display peptide library kit (New England Bio-labs) was used by following bio-panning approaches established so far [22]. In this approach, bio-

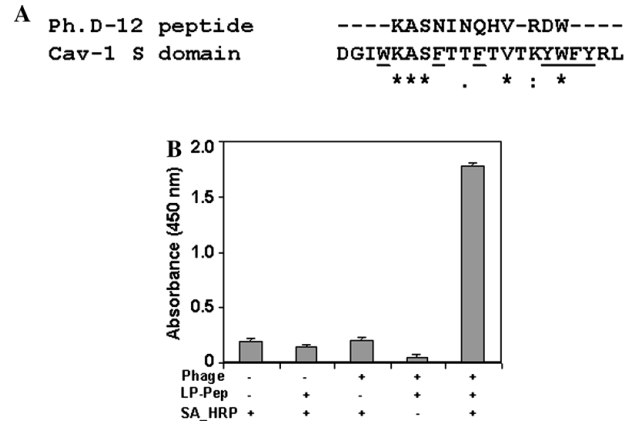

Fig. 1. (A) ClustalW alignment of peptide selected from phage library. The peptide sequence obtained against the biotinylated α-HL-LP peptide, denoted by "Ph.D-12 peptide," is aligned with the scaffolding domain of Caveolin-1, "denoted by Cav-1 S domain." The alignment was done as per the web site (http://www.ch.embnet.org/software/ClustalW.html). In the Caveolin-1 scaffolding domain, the positively charged residues are K and R and the aromatic residues are underlined. (*) A perfect match; (:) represents substitutable residue, and (·) represents similar residue. (B) Interaction of biotinylated LP peptide with the purified phage. The phage clone, eluted after biopanning and screening, was cultured, coated in 96-well plates overnight. After removal of unbound phage, the wells were blocked and ELISA was carried out with biotinylated LP peptide. The bound LP peptide was detected with the help of streptavidin-HRP and color development was with ABTS. The data represent the average of three independent measurements.

tinylated-LP peptide of α-HL (Biotin-QN**WGPYDR DSW**NPVY) which contains the Caveolin-1 binding motif has been used. The data in Fig. 1A show the peptide sequence obtained after three rounds of selection. Clustal W alignment of this peptide with scaffolding domain of Caveolin-1 resulted in noticeable similarity (Fig. 1A). This finding was in agreement with the ELISA, which had shown 4–6-fold increase in OD_{450}, in comparison to the controls (Fig. 1B). We have also observed the interaction between the LP peptide of α-HL and the scaffolding domain of Caveolin-1 by ELISA (data not shown).

Surface plasmon resonance studies

In view of the above result, we then examined whether Caveolin-1 can interact with α-HL and inhibit the hemolysis of rabbit red blood cells. In these experiments, recombinant Caveolin-1 was incubated with native α-HL at various ratios and this complex was added to rabbit red blood cells to monitor the hemolysis. The data in Fig. 2A clearly show a decrease in hemolysis with an increase in Caveolin-1 concentration. We could not achieve 100% inhibition of lysis probably due to the oligomeric nature of Caveolin-1, which exists as filaments under in vitro conditions (data not shown). Nevertheless, the data lend support to our observation that

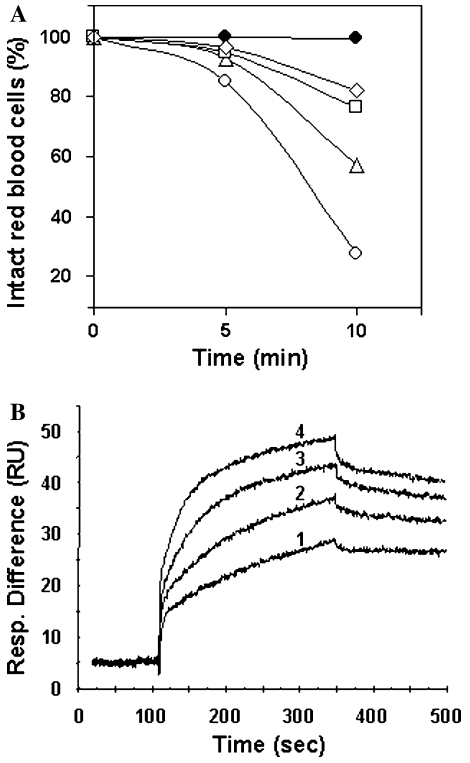

Fig. 2. (A) Inhibition of α-HL induced hemolysis by Caveolin-1. In this hemolysis assay, we have incubated α-HL and different ratios of Caveolin-1 and examined the rabbit red blood cell lysis. Filled and open circles, respectively, represent control rabbit red blood cells and α-HL alone. The various ratios of α-HL: Caveolin-1 are 1:2 (△), 1:4 (□), and 1:6 (◇). The data represent the average of three independent measurements. (B) Surface plasmon resonance response curves for the interaction of α-HL and Caveolin-1 scaffolding domain. Biotinylated scaffolding domain of Caveolin-1 is immobilized at 500 RUs on a streptavidin chip supplied by the manufacturer. Highly purified α-HL was diluted in HBS-EP buffer (100 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20, and 0.02% sodium azide) and passed at a flow rate of 30 μl/min through flow cells 1 and 2, where flow cell 1 is blank surface. After obtaining a good baseline various concentrations of α-HL are passed through the chip at about 120s as indicated by the numbers (1) 125 nM; (2) 250 nM; (3) 500 nM, and (4) 1000 nM. Dissociation phase was obtained by passing buffer alone which was at about 350 s. The data represent one of three independent sets of measurements.

the motif in α-HL is capable of interacting with the Caveolin-1. In order to localize the interaction between α-HL and Caveolin-1 further, we have used surface plasmon resonance technique to study the specificity of this interaction. The data shown in Fig. 2B clearly show a concentration dependent interaction between α-HL and the immobilized Caveolin-1 scaffolding domain peptide in HBS-EP buffer. The sensograms can be fitted to a two-step process. A noticeable feature is the dissociation of α-HL which is rather slow ($k_{-1} \sim 10^{-4}\,s^{-1}$) indicating the tightness of the binding between α-HL and the scaffolding domain of Caveolin-1. Based on the sensograms shown in Fig. 2, it is possible that the first step may be due to an electrostatic (ionic or salt bridge) interaction between the α-HL and the scaffolding domain of Caveolin-1. This could be because the scaffolding domain of Caveolin-1 contains three positively charged residues (basic residues K and R in Fig. 1A), while the motif of α-HL has two negatively charged residues (D^{183} and D^{185}).

We then examined the role of this nine amino acid sequence in the folding and function of α-HL. From the crystal structure of α-HL, it is clear that these nine amino acids are not involved in interprotomer interactions [2]. In addition, the loop containing the nine amino acids appears to be highly flexible and hence can be modified without affecting the backbone and overall folding of α-HL. In view of the flexibility, we chose to delete the nine amino acid motif. A deletion approach rather than creating single point mutations at this location was adopted since the aromatic to glycine substitutions (Φ to G) in the EGFr Caveolin-1 binding motif had not resulted in abolishing the interaction between EGFr and Caveolin-1. The mutations still resulted in the inhibition of kinase activity of EGFr [17]. In addition, in the absence of a clear picture of interactions, i.e., π–π stack, or hydrophobic or electrostatic interactions coupled with the role of this loop in α-HL monomer, point mutations may not have yielded conclusive results. Therefore, we shortened the loop of α-HL containing the Caveolin-1 binding motif by deletion thereby eliminating all the possible interactions. Two deletion mutants of α-HL viz. α-HL-SD (short deletion of the loop) and α-HL-LD (long deletion of the loop) in which 9 (179–187) and 35 (179–213) amino acids were, respectively, replaced with Gly-Ser sequence were made to examine the role of this loop in the folding and function of α-HL. Both mutants were expressed in *E. coli* and purified to homogeneity as shown in Fig. 3A. The properties of α-HL-SD and α-HL-LD mutants were examined by: (i) limited proteolysis to understand the domain architecture of α-HL, (ii) intrinsic fluorescence studies to examine the nature of the surrounding area of the motif, and (iii) the role of this motif in assembly and pore formation by hetero-oligmerization studies.

Limited proteolysis by proteinase K

Limited proteolysis of α-HL by proteinase K yields a two 'half-like' pattern and these resultant two halves are fairly resistant to further proteolysis by proteinase K. Based on these data, it was inferred that the α-HL is composed of two rigid domains linked by a glycine rich loop, which inserts into the membrane [23]. In case of wild-type α-HL, proteinase K susceptible peptide bonds are between 131 and 136 amino acid residues. When α-HL-SD and α-HL-LD were subjected to proteolytic cleavage by proteinase K, we could detect the two half-like patterns similar to that of α-HL (Fig. 3B and please refer to [18] for the pattern of α-HL). A glance at the Fig. 3B reveals that, although the pattern is the same as that of α-HL, the time points

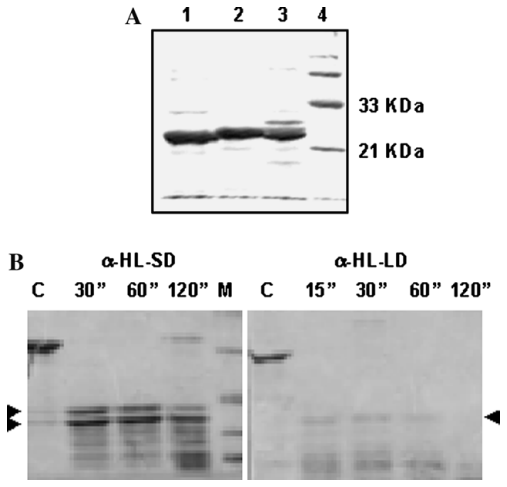

Fig. 3. (A) Purified recombinant α-HL, α-HL-SD, and α-HL-LD. The recombinant constructs were expressed in *E. coli* BL21 (DE3) and the respective inclusion bodies were isolated and processed as described in Materials and methods section. Various lanes represent α-HL-LD (1), α-HL-SD (2), and α-HL (3) and low molecular weight markers (4). We have consistently noted that the α-HL-SD has slightly abnormal mobility, i.e., it migrates slower than α-HL and α-HL-LD migrates slightly ahead of α-HL. (B) Proteinase K digestion pattern of α-HL-SD and α-HL-LD: α-HL-SD or α-HL-LD (16 μg) was incubated with 250 ng of proteinase K at room temperature. Aliquots were taken at indicated times into pre-heated (95 °C) tubes containing 1× LSB and phenylmethylsulfonyl fluoride and electrophoresed. Following electrophoresis, the gel was stained with Coomassie blue. C, represents control protein, M represents low molecular weight markers.

of digestion are in the range of a few seconds instead of several minutes usually observed for native α-HL. Between α-HL-SD and α-HL-LD, the latter mutant appears to be more susceptible to proteinase K as we could not observe a stable lower half of α-HL (lower band). We suspect that the α-HL-LD is more prone to further proteolysis because of exposed segments. Nevertheless, these data clearly suggest that the overall folding of α-HL-SD and α-HL-LD is very much similar to that of α-HL (i.e., the first cut by proteinase K) but the mutants are highly susceptible to further proteolysis by proteinase K, unlike α-HL. This observation is in sharp contrast to a carboxy terminal deletion mutant (α-HL(1–289)) which was completely digested by proteinase K at all time points because of incomplete folding [18]. This suggests that the nine amino acid motif may provide stability to the domain (carboxy terminal) of native α-HL, resisting the proteolytic attack by proteinase K.

The overall secondary structures, as examined by near- and far UV-circular-dichroism spectra of α-HL-SD and α-HL-LD, are similar to that of native α-HL ([15,18,23]; data not shown). Based on the CD data as well as the proteolytic cleavage profile, it is reasonable to conclude that both α-HL-SD and α-HL-LD have similar overall folding to that of native α-HL.

Intrinsic fluorescence emission studies

The intrinsic fluorescence emission (IFE) of α-HL-SD and α-HL-LD was examined because we had deleted two tryptophan residues and a tyrosine residue in these mutants. The data in Fig. 4A are a representative profile of IFE spectra of α-HL, α-HL-SD, and α-HL-LD which show that the emission maxima of the α-HL-SD and α-HL-LD almost remain unaltered (337 nm) in comparison to α-HL (336 nm; [18]). This finding indicates that the deletion in primary structure did not lead to massive alteration in the solvent accessibility of tryptophan residues of water-soluble monomer of α-HL (for comparison the α-HL, α-HL-SD, and α-HL-LD have 8, 6, and 6 tryptophan residues, respectively). However, the motif deficient mutants have exhibited a significant increase in fluorescence intensity at the same protein concentration (i.e., 30 μg/ml), suggesting that the tryptophan residues

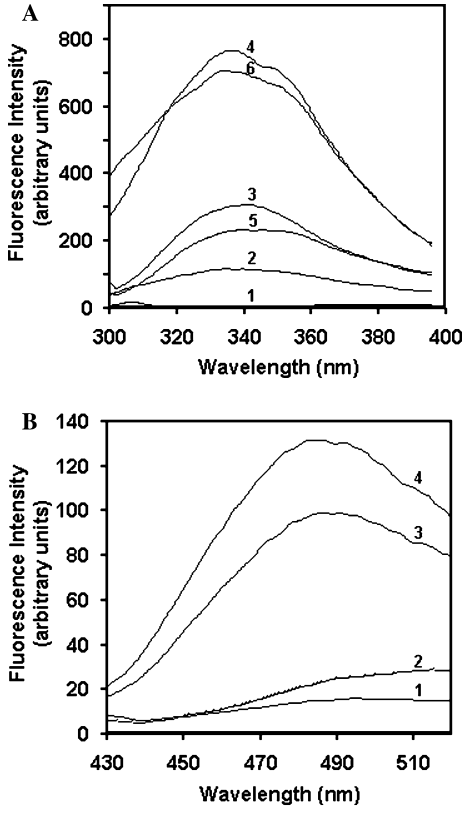

Fig. 4. (A) IFE of α-HL, α-HL-SD, and α-HL-LD mutants. Purified proteins (30 μg/ml) in Mops buffer, pH 7.0, were equilibrated at 25 °C in 1 cm path length quartz cuvettes. IFE was observed after excitation of the sample at 280 nm or 295 nm and the emission was collected above 330 nm. The various spectra represent the fluorescence intensity of buffer (1), α-HL (2), α-HL-SD (3), and α-HL-LD (4) at 280 nm excitation and α-HL-SD (5) and α-HL-LD (6) at 295 nm excitation. All spectra were corrected for buffer and scattering. (B) Enhancement of ANS fluorescence. Enhancement of ANS fluorescence was observed after addition of a 30 μl of ANS to α-HL, α-HL-SD, and α-HL-LD mutants in Mops buffer, pH 7.0, as reported earlier [18]. The fluorescence intensity of ANS in presence of buffer (1), α-HL (2), α-HL-SD (3), and α-HL-LD (4).

in these mutants are more exposed. We had earlier reported that the α-HL upon complete denaturation exhibited emission maxima at 356 nm. In comparison, α-HL (1–289), which was completely digested by proteinase K, had exhibited emission maxima at 345 nm. Based on the emission maxima observed for α-HL, α-HL (1–289), and denatured α-HL, it is reasonable to assume that the motif deletion mutants of α-HL are fairly well folded.

Exposure of hydrophobic pockets

1-Anilino-8-naphthalene sulfonic acid (ANS) is conventionally considered to bind to preexisting hydrophobic (nonpolar) surfaces of proteins through its nonpolar anilinonaphthalene group. ANS has been used extensively to characterize the unfolding characteristics and study of hydrophobic cores of proteins [24]. Binding of ANS to the proteins occurs only upon the exposure of hydrophobic patches/clusters during the unfolding and refolding process [18,25]. To compare the relative hydrophobic surface area between the wild type α-HL and its motif deletion mutants, we performed the ANS binding experiments. The ANS fluorescence emission was practically unchanged when titrated with α-HL, suggesting that α-HL at neutral pH does not bind ANS. But in case of α-HL-SD and α-HL-LD mutants the fluorescence intensity increased dramatically (by about 6–7-fold), and the λ_{max} was blue-shifted by about 40 nm (Fig. 4B). These spectral changes, in addition to the increase in fluorescence intensity, imply that, both these mutants contain hydrophobic sites that are clearly exposed unlike intact α-HL, at neutral pH.

Hetero-oligomerization studies

Among many in vitro studies, hetero-oligomerization studies of α-HL are very useful and informative. In this assay, the mutants of α-HL are evaluated for their ability to get incorporated into the heptamers formed by native α-HL. A given labeled mutant has undergone conformational changes on the cell surface or not can be studied by allowing the mutant to bind the target cells first and then chased with the wild type unlabeled α-HL, due to the fact that, α-HL oligomerization on cell surface occurs among the monomers that have undergone conformational changes in a similar way. We have generated [^{35}S]methionine forms of these mutants by in vitro transcription and translation, incubated with rabbit red blood cells, and chased with unlabeled α-HL. In such experiments, we have not been able to detect any hetero-oligomeric band of α-HL-SD and α-HL-LD with α-HL despite overexposure of the autoradiogram (Fig. 5A). Moreover, these mutants do not form homo-oligomers on both rabbit red blood cells and A431 cells (data not shown). We have also used the rabbit red blood cell

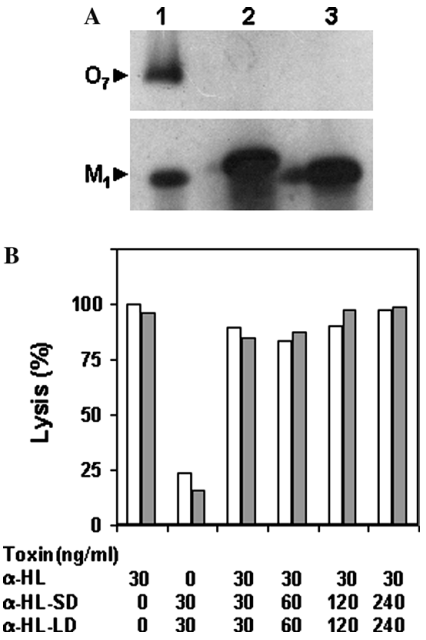

Fig. 5. (A) Absence of hetero-oligomers for α-HL-SD and α-HL-LD. In this experiment, we have generated [^{35}S]met labeled toxins of α-HL, α-HL-SD, and α-HL-LD mutants. [^{35}S]Met labeled α-HL-SD and α-HL-LD were first allowed to bind to rabbit red blood cells (30 min) and incubated for an additional 30 min with unlabeled α-HL. At the end of the lysis, the RBCs were pelleted by centrifugation and electrophoresed for autoradiography. Lane 1 represents control [^{35}S]met labeled α-HL alone. Lane 2 and lane 3 represent α-HL-SD and α-HL-LD, respectively. (B) Absence of dominant negative effect of α-HL-SD and α-HL-LD on α-HL induced hemolysis. In this assay, α-HL and α-HL-SD and α-HL-LD were mixed with increasing ratios as indicated and incubated with rabbit red blood cells. Absorbance was measured at 595 nm, after 30 min of incubation time. The data represent the average of four independent measurements.

lysis assay to understand whether α-HL-SD and α-HL-LD can block or enhance the red blood cell lysis by native α-HL. The data shown in Fig. 5B clearly show that these motif deficient mutants do not have the ability to block the lysis by α-HL, in contrast to the amino terminal deficient mutant shown to inhibit the lysis of α-HL, exhibiting a dominant negative character (10). Moreover, several other point mutations in the central region have been shown to exhibit dominant negative and positive character [26]. Interestingly, α-HL(1–289) also did not get incorporated into the oligomers of wild type α-HL [18]. It can be argued that in case of α-HL-SD and α-HL-LD also, the same may be true, i.e., these mutants have partially unfolded. However, the CD data (data not shown), proteolytic digestion profile (Fig. 3B), and IFE (Fig. 4A) clearly demonstrate that these mutants have near normal folding except that their hydrophobic core is exposed. If the Caveolin-1 binding motif was not important for undergoing initial conformational changes on erythrocytes or nucleated cells (e.g., A431), then one would expect the α-HL to assemble on erythrocyte surface irrespective of the presence of the motif. Since, its hydrophobic core was intact as well as the same

phospholipids, e.g., PC and unsaturated PC were also present on erythrocytes as well. In other words, we should have seen heptameric assembly for the α-HL-SD and α-HL-LD mutants, albeit weak, oligomeric bands, but we have not been able to detect any oligomeric band for these mutants (oligomerization process is driven by the strength of hydrophobic interactions with cell surface coupled with the strength of interprotomer interactions. Please note that each α-HL protomer participates in 850 van der Waals contacts and about 120 salt bridges and ionic interactions in the heptameric form of α-HL). All these observations suggest that the conformational changes in α-HL required for oligomerization are probably triggered at this stretch of amino acids. In the absence of this motif sequence, the α-HL may not undergo conformational changes. Granulocytes have been shown to be insensitive to α-HL attack [12]. It was concluded that the 14-strand β-barrel failed to penetrate the granulocyte membrane. It has also been reported in the literature that granulocytes do not express Caveolin-1. This observation, in conjunction with our data, explains that the failure of the β-barrel of α-HL in penetrating the granulocyte cell membrane could be due to the absence of Caveolin-1.

In summary, we have been able to demonstrate that the α-HL has an amino acid sequence that interacts with Caveolin-1 specifically. The identified sequence is essential for maintaining the domain architecture of α-HL, i.e., in the absence of this sequence, the "half-like" domains of α-HL are highly susceptible to proteinase K unlike α-HL. Another interesting aspect is that in the absence of this sequence, the hydrophobic core of α-HL is exposed. This suggests that this water-soluble sequence shields the hydrophobic pocket of α-HL from bulk solvent. For pore formation, the K266 residue in α-HL was shown to insert into the membrane bilayer along with the 14-strand β-barrel. The sequence identified by us lies in between the K266 (outer periphery) and the β-barrel of α-HL. Hence, during pore formation, this sequence also has to penetrate the membrane bilayer. In fact, studies employing site-directed chemical modification of D183C, R184C, and D185C mutants have suggested that this stretch of amino acids might penetrate the membrane bilayer [27]. In the light of these observations, we propose that the α-HL may interact with Caveolin-1 of mammalian cells for pore formation. In addition, the mutants demonstrated here may serve as good starting steps in defining the nature of interactions that take place at cell surface.

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