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Biochemical and Biophysical Research Communications 363 (2007) 197–202
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Aromatic residues of Caveolin-1 binding motif of α-hemolysin are essential for membrane penetration

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Received 16 August 2007
Available online 30 August 2007

Abstract

We have created *s*ingle *c*ysteine *C*aveolin-1 *b*inding *m*otif *m*utants (SCCBMMs) of staphylococcal α-HL for understanding assembly and penetration. All SCCBMMs have normal folding like α-HL as examined by limited proteolysis, intrinsic fluorescence emission, no hemolytic activity and do not form hetero oligomers with α-HL indicating that the conformational changes occurred at the cell membrane are different to that of α-HL. While modification of SCCBMMs with a membrane impermeant reagent has resulted in reduced binding, badan modification has resulted in the enhancement of badan fluorescence with time of assembly (incubation time) indicating the change in environment of the badan and the need for the penetration of the aromatic amino acids. Our studies indicate that the conformational changes are probably initiated at the Caveolin-1 binding motif and provide a basis for differential mode of interaction of the Caveolin-1 binding motif depending upon the nature of the target cell membrane.

Keywords: α-Hemolysin; Caveolin-1 binding motif; Scaffolding domain; Hetero-oligomerization; Membrane penetration

Staphylococcal α-hemolysin (α-HL), a pore forming toxin, can assemble on erythrocytes, human monocytes, lymphocytes, platelets, and endothelial cells but with varied degree of susceptibility [1,2]. The assembly of α-HL (nucleated or non-nucleated) on target cells is proposed to include at least three distinct stages viz. (i) the binding of water soluble monomers to target membrane, (ii) oligomerization of the membrane bound monomer into a heptameric pre-pore, and (iii) the final conversion of the pre-pore into a functional pore [3]. The susceptibility of target cells can vary over many orders of magnitude suggesting the existence of a 'receptor' that might facilitate its assembly. We have earlier provided some biochemical and modeling based evidences in which we have shown that the α-HL can assemble on mammalian cells with the help of functional form of Caveolin-1, the major structural protein of caveolae (flask shaped invaginations) present on mammalian cell membrane and its function is dependent on cholesterol [3–6]. In addition, the α-HL cannot assemble on cell membranes devoid of Caveolin-1 such as HT29 colon cancer cells and human granulocytes [4,7].

The interaction between α-HL and Caveolin-1 was suspected to occur through the scaffolding domain amino acids 81–101 of Caveolin-1 and the Caveolin-1 binding motif amino acids 179–187 of α-HL through likely ionic and π–π stack interactions [4]. This mode of binding might initiate some conformational changes for an efficient insertion of its transmembrane β-barrel. But the question remained unsolved as to how α-HL can establish contact with the Caveolin-1 which is present in the cytoplasmic face of membrane? For facile penetration, the Caveolin-1 binding motif should have high propensity to insert into lipid bilayer. Hence, it is important to understand the membrane penetration ability of the Caveolin-1 binding motif during the assembly of α-HL. Such information can help us to generalize, whether or not the Caveolin-1 binding motif sequences of other toxins/proteins can penetrate eukaryotic cell membranes for defining the pathological consequences.

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doi:10.1016/j.bbrc.2007.08.132

Materials and methods

Site directed mutagenesis, protein expression and purification. The following mutants W179C, Y182C, and W182C (together referred as *s*ingle *c*ysteine *C*aveolin-1 *b*inding *m*otiff *m*utants (SCCBMMs)) of α-HL were constructed by site directed mutagenesis using the Quick-Change kit (Stratagene). Plasmid containing full length 6-His-α-HL in pET28a^{+} vector (Novagen) was used as template for mutagenesis and all mutations were confirmed by DNA sequencing. Bacterial expression and purification is carried out as reported earlier [6,8].

Limited Proteinase K digestion. Purified mutants (30 μg) were incubated with 500 ng Proteinase K and at different time points, an equivalent of 4 μg of digested mutant removed and processed for SDS–PAGE as reported earlier [6].

Measurement of intrinsic and ANS fluorescence. Fluorescence emission spectra in presence and absence of 1-anilino-8-naphthalene sulfonic acid (ANS) of the mutants were acquired with Perkin–Elmer LS50B spectrofluorimeter at 25 °C by using 30 μg/ml of protein in MOPS buffer (10 mM MOPS, pH 7.4) as reported earlier [6]. The excitation wavelength was 280 nm for intrinsic fluorescence and for ANS studies it was 380 nm. The slit width was 5 nm for all measurements.

Kinetics of hemolysis of rRBCs by α-HL and SCCBMM. Purified SCCBMMs (5 μg/ml) were incubated (in presence or absence of DTT) in 200 μl of 1% rRBC in KPBSA. At regular intervals of time, light scattering was recorded at 595 nm as reported earlier [8].

Binding, oligomerization, and oligomer stability of SCCBMM on rRBCs. For binding and oligomerization assay, SCCBMMs (5 μg/ml) and α-HL (1 μg/20 μl) were incubated in 0.5% of rRBCs for 1 h and membranes were collected by centrifugation at 1600 rpm and resolved by 7.5% SDS–PAGE [6,8]. For oligomer stability assay, instead of 5 μg/ml, 10 μg/ml protein was used for incubation and heated at 50 °C or 65 °C for 10 min.

Digestion of membrane-bound SCCBMM with Proteinase K. α-HL or SCCBMMs (5 μg/ml) were incubated for 15 min or 1 h in 1 ml rRBCs (1%) and membranes were isolated by centrifugation. The membrane pellet was resuspended in 20 μl of KPBS and incubated with Proteinase K (1 μg). Reaction was stopped by the addition of 1× laemmli's sample buffer containing 1 mM PMSF, heated at 95 °C, 5 min and subjected to 15% SDS–PAGE.

Hetero-oligomerization assay. Purified mutants were incubated with wild type α-HL with indicated molar ratios exactly as reported earlier [6].

IASD and badan modification of SCCBMMs. Each SCCBMM was labeled with 4-acetamido-4′-((iodoacetyl) amino) stilbene-2, 2′-disulfonate (IASD) or badan at 1:5 molar ratio at room temperature for 1 h in presence of 10 mM DTT as reported earlier [9]. Quantitative analysis of binding, oligomerization and kinetics of lysis of IASD modified proteins were examined on RBC membrane using 5 μg/ml labeled and unlabeled protein as described earlier [8]. For membrane penetration, measurement after badan modification, 5 μg of labeled SCCBMMs were incubated for 6 h in 1 ml 0.25% RBCs in dim light, washed three times with KPBSA and excited at 408 nm and emission spectra was recorded between 400 and 600 nm. For controls spectra were recorded immediately after addition to rRBCs.

Results

Cloning, expression and purification of SCCBMMs

We expressed α-HL and all SCCBMMs with a carboxy terminus 6-histidine tag for easy purification (Fig. 1A) as extension of α-HL's carboxy terminus does not affect the overall folding and assembly of α-HL [10].

SCCBMMs do not lyse target cells

Interestingly, none of the SCCBMMs were able to lyse rRBCs in 24 h even at a concentration of 5 μg/ml as α-HL requires only 20 ng/ml in a few minutes (data not shown). The data in Fig. 1B shows that the DTT treatment did not result in eliciting the hemolytic activity of SCCBMMs. In summary, point mutations in Caveolin-1 binding motif of α-HL abolished the hemolytic activity not due to intermolecule disulfide bond formation but the aromatic residues probably play a decisive role in membrane penetration during the assembly of α-HL on target cells.

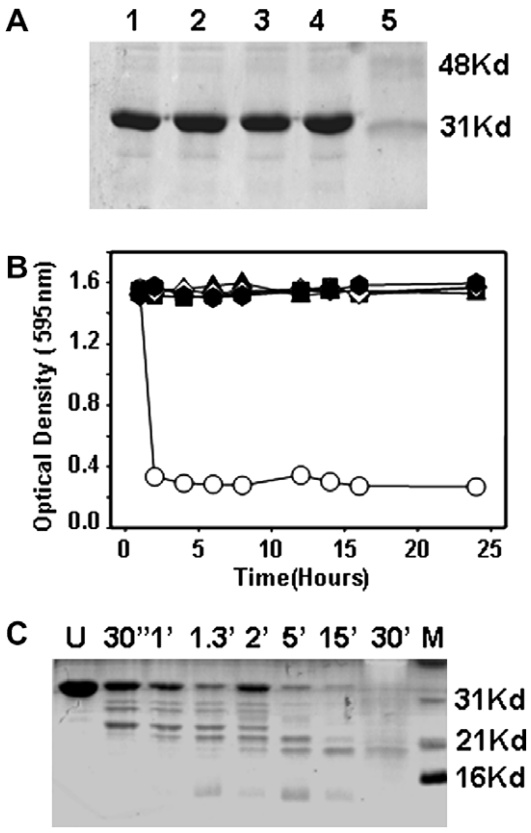

Fig. 1. (A) Purified recombinant 6-His-α-HL and SCCBMMs: The recombinant constructs were expressed in BL21 (DE3). Respective soluble fractions were prepared and processed as described in Materials and methods section and purified using Ni-NTA agarose. Lane 1: 6-His-α-HL, lane 2: W179 C, lane 3: Y182C, lane 4: W187C, lane 5: low molecular weight markers (BioRad). (B) Quantitative hemolysis assay of α-HL and SCCBMMs in presence of DTT: Final concentration of each protein was 5 μg/ml diluted in 200 μl 1% rRBCs and lysis was measured at 595 nm in presence of 5 mM DTT. The data represents the average of three independent measurements. Symbols corresponds to α-HL (○), W179C (■), Y182C (●), and W187C (♦). (C) Susceptibility of SCCBMMs to Proteinase K: Each SCCBMM (30 μg) in solution was treated with 500 ng of Proteinase K as described in Materials and methods section. The samples were resolved by 15% SDS–PAGE and stained with coomassie brilliant blue. A representative panel for W179C is shown. The lanes labeled with U and M represents the undigested protein and low range proteins markers, respectively.

Limited proteolysis by Proteinase K of SCCBMMs

Proteinase K cleavage of α-HL results in a two-half like domains (primary cleavage occurs between 131 and 136 amino acids) and very useful for understanding the overall

folding of α-HL. Based on this logic, the relative overall folding of SCCBMMs were evaluated by limited proteolysis. From Fig. 1C, it is clear that all SCCBMMs (data shown for W179C only) have nearly similar folding like α-HL as they have yielded the characteristic two half like pattern. However, subtle differences exist between the SCCBMMs. For example, W179C is more susceptible than the other two mutants. These observations are consistent with our earlier report in which we have created α-HL mutants devoid of Caveolin-1 binding motif which were highly susceptible to Proteinase K [6].

In vitro stability of SCCBMMs

To access relative folding of SCCBMMs, the intrinsic fluorescence emission (IFE) was examined as we converted two tryptophans (179 and 187) and one tyrosine (182) into cysteine in case of SCCBMMs. The data in Fig. 2A is a representative profile of intrinsic fluorescence emission spectra of α-HL and three SCCBMMs. Over all, only negligible red shift in the emission maximum was observed for all SCCBMMs. Specifically W179C and Y182C showed ~1 nm shift (at 337 nm instead of 336 nm observed for α-HL) and 2.5 nm shift was recorded for W187C (~338.5 nm) in comparison to α-HL at 336 nm [6,10]. From these data it is clear that no massive conformational or structural destabilizations were observed due to the point mutations at the Caveolin-1 binding motif. We had earlier reported that the α-HL upon complete denaturation exhibited an emission maximum at 356 nm. In comparison, α-HL(1–289) (devoid of 4 carboxy terminal amino acids) had exhibited an emission maxima at 345 nm and was completely digested by Proteinase K (no two half like pattern, Ref. [10]). Hence, it is reasonable to assume that the SCCBMMs described here are fairly well folded and very much similar to α-HL. The surface hydrophobicities of the α-HL and SCCBMM were compared by their ability to bind ANS as it binds only to hydrophobic pockets, if any, of proteins. The ANS fluorescence emission was unchanged when compared with α-HL suggesting that SCCBMMs at neutral pH do not bind ANS (data not shown). Thus, point mutations in this region do not affect the overall surface hydrophobicity where as deletion does [6]. This clearly indicates that the Caveolin-1 binding region of α-HL acts as water insulator to hydrophobic residues that bind to ANS after removal of Caveolin-1 binding motif containing loop [6]. Hence, modifications of Caveolin-1 binding motif, whether point mutation or loop deletions do not affect the global folding of α-HL and mutants are fold like native α-HL.

Mutations do not abolish SCCBMM binding to target cells

In view of the loss of hemolysis of SCCBMMs, we examined whether or not lack of hemolysis was due to impairment in efficient binding and/or oligomerization and/or membrane penetration. Interestingly, all the

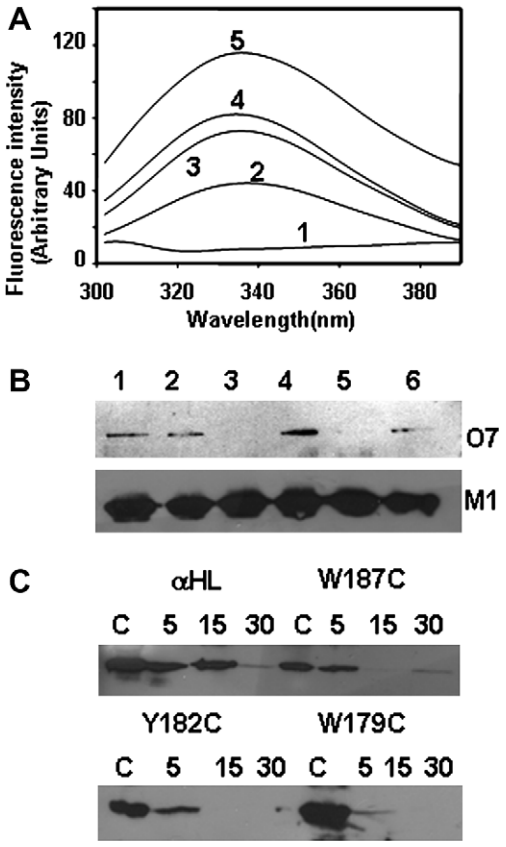

Fig. 2. (A) Intrinsic fluorescence of α-HL and SCCBMMs: α-HL and SCCBMMs (30 μg/ml) in MOPS buffer (10 mM, pH 7.4) were equilibrated at 25 °C in 1 cm path length quartz cuvettes and the fluorescence intensity was recorded in a Perkin–Elmer LS50B spectrofluorimeter. All samples were excited at 280 nm and the emission spectra were recorded from 300 to 400 nm. All measurements were averaged over three scans. The various spectra represent the fluorescence intensity of buffer (1), Y182C (2), W179C (3), W187C (4), and α-HL (5). All spectra were corrected for buffer and scattering. (B): Heat stability of SCCBMM oligomers: Binding and oligomerization to rRBCs was performed by incubation of 10 μg/ml of each SCCBMM mutant with rabbit erythrocytes (1% RBCs) for 1 h at room temperature as described in Materials and methods section. Samples were either heated at 50 or 65 °C with 1× laemmli's sample buffer and the rest of the procedure is as described above. Lane 1: W187C (50 °C), lane 2: W187C (65 °C), lane 3: Y182C (50 °C), lane 4: Y182C (65 °C), lane 5: W179C (50 °C), and lane 6: W179CC (65 °C). (C) Proteinase K digestion of membrane bound α-HL and SCCBMMs: The toxin-treated membranes were digested with Proteinase K (0.05 mg/ml) as described in Materials and methods section. Samples were electrophoresed on 15% SDS–PAGE and transferred to nitrocellulose paper followed immunoblot with anti-his tag antibody. C represents the control (without Proteinase K treatment), and the numbers represent the time length of Proteinase K treatment in minutes.

SCCBMMs bound to target membranes and oligomerized but with certain degree of variability (Fig. 2B). Among the SCCBMMs, W179C showed highest binding but lowest oligomerization where as W187C showed lowest binding but efficient oligomerization (data not shown). Oligomers formed by the mutants are stable at 50 °C and 1% SDS like its wild type counterpart which are stable upto 65 °C in 1% SDS [3]. Noticeably, the oligomers of SCCBMMs showed reduced stability (Fig. 2B) with the exception of the

oligomer formed by W187C which behaved like α-HL. In view of absence of lysis and oligomer formation, the oligomer bands can be classified as pre-pore oligomers. In principle, the pre-pore can be subdivided into three categories viz. (i) A pre-pore which is closer to the membrane bound monomer, (ii) a pre-pore closer to the functional pore, and (iii) an intermediate between (i) and (ii). For example, the H35C mutant appears to belong to category (ii) while the H35N mutant can be classified into category (i). In our observation, the pre-pores formed by the SCCBMMs belong to category (iii) based on the data shown in Fig. 2B and our unpublished data. Hence, it is clear that the SCCBMMs could not fulfill the final step for pore formation and the mutated aromatic residues play an important role at later stage of pre-pore formation also in addition to the membrane binding.

Proteolytic stability of SCCBMMs on RBCs membrane

α-HL, which assembled partially or arrested at pre-pore stage, is susceptible to cleavage at its N-terminus by Proteinase K. Based on resistance to cleavage at the amino terminus, it is possible to identify whether or not an α-HL mutant proceeded beyond pre-pore stage. The proteolytic susceptibilities of SCCBMMs were compared with α-HL and H35N in presence of RBC membrane (Fig. 2C). The time dependent proteolytic resistance was found to be in the order α-HL ⩾ W187C > Y182C > W179C ⩾ H35N. All SCCBMMs (except W179C) assembled beyond the susceptible pre-pore stage as they are quite resistant to Protinase K. W179C which showed highest susceptibility to Proteinase K, probably assembled like H35N mutant. It is probable that the mutants form a pre-pore like intermediate, which differ in terms of degree of inter-protomer interactions. This data clearly indicates that the Caveolin-1 binding motif has a significant role in membrane penetration for functional pore formation.

SCCBMMs do not form hetero-oligomers

We have previously shown that Caveolin-1 binding motif directly involved in oligomerization of α-HL and its deletion totally abolished the co-operative oligomerization property [6,11]. All SCBMMs oligomerized reasonably well, however, surprisingly, could not form hetero-oligomers with α-HL (Fig. 3A). The lysis caused by α-HL was not affected by an increase in SCCBMM concentration. It is also clear that the co-operative oligomerization is defective in case of SCCBMMs. Interestingly, the oligomers formed by the SCCBMMs, to a certain degree, are fairly resistant to heat and SDS which is a characteristic property of α-HL.

IASD modification of SCCBMMs and topology

Utility of IASD has been well established for the study of topology of membrane binding proteins [9,12]. IASD

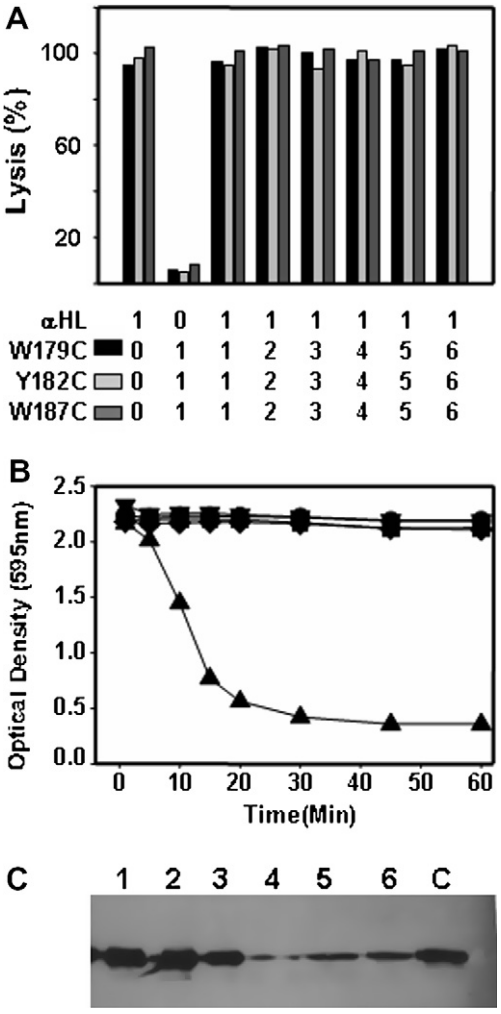

Fig. 3. (A) Hetero-oligomerization assay: Hetero-oligomerization assay in the presence of wild type α-HL as described in Materials and methods section. Increasing molar concentration of SCCBMM was incubated with α-HL as indicated in 1 ml of 1% rRBCs. The percentage lysis caused by mixture proteins was measured as reported earlier [6]. The data represents the average of four independent measurements. (B) Kinetics of lysis with IASD labeled SCCBMMs: Each SCCBMM was incubated in 0.05 M sodium phosphate, pH 7.5, containing 10 mM DTT for 5 min at room temperature in the molar ratio of 5:1 as described in Materials and methods section. Activity of IASD labeled proteins were examined as described in Fig. 1 in presence of 5 mM DTT. Symbols correspond to α-HL (▼), W179C-IASD (▲), Y182C-IASD (●), and W187C-IASD (♦). (C) RBC membrane binding and oligomerization with IASD labeled proteins: SCCBMMs were labeled with IASD as described above and the membrane binding and oligomerization assay was carried out as done for Fig. 2B by using 5 μg/ml of IASD labeled or unlabeled protein as control. The binding represents as W179C (lane 1), Y182C (lane 2), W187C (lane 3), W179C-IASD (lane 4), Y182C-IASD (lane 5), and W187C-IASD (lane 6). Lane C represents α-HL as marker. (B) and (C) were obtained with anti-6-his antibody.

labeled SCCBMMs showed no lytic activity (Fig. 3B) and reduced membrane binding (Fig. 3C) and no oligomerization (data not shown) in contrast to IDSD labeled cysteine mutants of the amino acids D^{183}-R-D^{185}. This suggests that aromatic amino acids of Caveolin-1 binding motif are involved in the binding and membrane penetration for β-barrel formation [12].

Membrane penetration of SCCBMMs

We have examined the membrane penetrability of the SCCBMMs by badan modification, which is an environmentally sensitive fluorophore. We have been able to achieve high labeling efficiency of all SCCBMMs (W179C (99.34%), W187C (98.45%), and Y182C (96.11%)) which indicates the high degree of accessibility of the aromatic amino acids to solvent (Fig. 4). Badan labeling did not change the silent nature of SCCBMMs towards lytic activity. We anticipated that badan can retrieve back the activity by fulfilling the aromatic surface requirement that was lost due to mutation (Fig. 4C). To determine relative penetration, badan emission spectra (emission spectra shift and increase intensity corresponds to membrane penetration) were recorded for all SCCBMMs after membrane binding. Interestingly, there was a marked increase in fluorescence emission intensity of badan as seen in Fig. 4D. All SCCBMMs have yielded blue shifts upon oligomerization but major shift was obtained for W187C. It is straightforward to interpret the apolar environment experienced by badan labeled mutants of the hydrophobic core of the lipid bilayer. All the data together indicate that the water soluble, Caveolin-1 binding motif of α-HL penetrates well inside the membrane to interact with Caveolin-1 or Caveolin-1 like molecule(s), plays an important role in oligomerization and conformational changes necessary to form transmembrane pore.

Discussion

Assembly of staphylococcal α-HL varies from cell type to cell type and this variation compels us to believe the existence of a receptor that might facilitate its assembly. The target cell susceptibility can vary as a function of the presence of Caveolin-1. However, Caveolin-1, *per se*, is absent on red blood cells of most species but enough evidence exits for its presence on mammalian cells [13,14]. The Caveolin-1 binding motif of α-HL appears to play a crucial role in its assembly. Most intriguing question that remained unanswered is how the α-HL establishes its contact with Caveolin-1, which is present in the cytoplasmic side of cell membrane?

Comparative study with previously reported Caveolin-1 binding motif deficient mutants (α-HL-SD, α-HL-LD) and a carboxy terminal deletion mutant, support the view that SCCBMMs are reasonably folded and Caveolin-1 binding motif is structurally flexible. All SCCBMMs appear to have normal binding to rabbit RBC membrane but have lost their co-operative oligomerization property, i.e., all SCCBMMs arrested as membrane bound pre-pore stage, although with varying degree of oligomer stability (Figs. 2C and 3A). These pre-pore oligomers have established a certain degree of inter-protomer contacts but not sufficient enough to form β-barrel assembly needed for cell lysis. This observation in conjunction with the absence of hetero-oligomer formation clearly indicates that the SCCBMMs have not undergone necessary conformational changes that

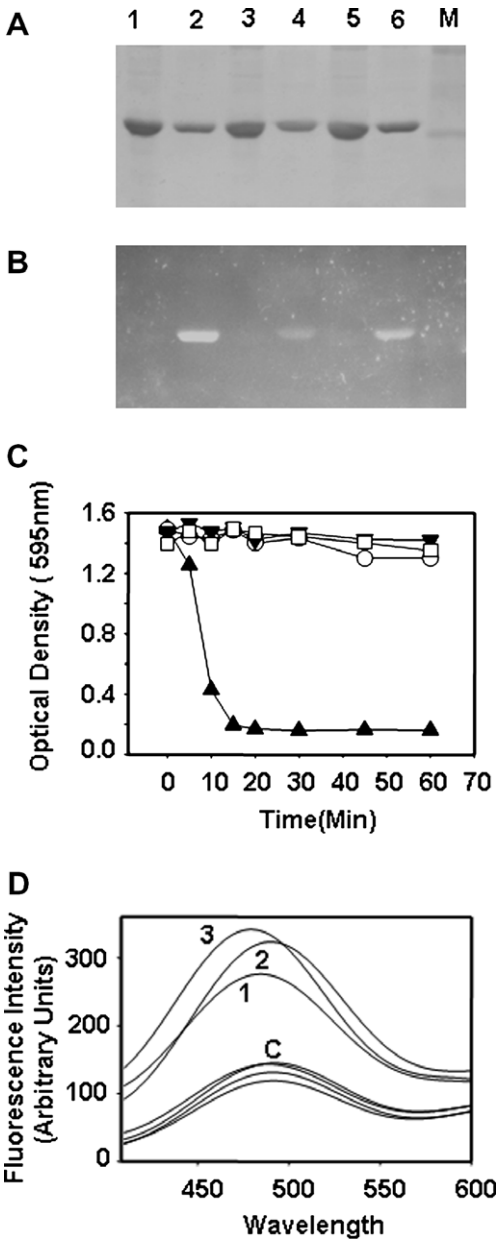

Fig. 4. (A,B) Badan modification of SCCBMMs. All SCCBMMs were modified with badan as mentioned in Materials and methods section and electrophoresed on 12% SDS–PAGE. The panel (A) represents the coomassie stained version of the gel and the panel (B) the badan emission detected with UV light. Lane 1: W179C, lane 2: W179C-Bd, lane 3: Y182C, lane 4: Y182C Bd, lane 5: W187C, and lane 6: W187C-Bd. (C) Hemolytic activity of badan labeled SCCBMMs was carried out as described for Fig. 1. The symbols respectively represent (□), (○), (▲), and (▼) for W179C, Y182C, W187C, and α-HL. (D) Relative emission spectra of badan labeled SCCBMMs on rRBCs were measured as described for Fig. 3A. Various spectra represents all labeled SCCBMMs at 0 min (C), W179C (1), Y182C (2), and W187C (3) after 6 h time point.

associate with the β-barrel penetration. In order to test the membrane penetration really needed for β-barrel formation we modified the SCCBMMs with badan, an environmentally sensitive fluorophore. Interestingly enough, we have observed differences in the intensity and blue shift of the badan emission signifying the change in the environment

of the aromatic residues of the Caveolin-1 binding motif of α-HL as membrane penetration is expected to result in an increase in emission intensity associated with blue shift (Fig. 4).

Cysteine scanning mutagenesis of charged residues of α-HL followed by site specific chemical modification of the cysteines by IASD have revealed that the charged residues of Caveolin-1 binding motif (W-G-P-Y-**D**-**R**-**D**-S-W) have interesting properties [12]. Firstly, single cysteine mutants have normal hemolytic activity but upon IASD modification have lost the same. This indicates that the penetration of D-R-D segment into the membrane is important. In contrast, the mutations at the aromatic residues of the Caveolin-1 binding motif (**W**-G-P-**Y**-D-R-D-S-**W**) led to loss of hemolytic activity (Fig. 1B). This clearly indicates that the aromatic residues play an important role in establishing 'apt cellular contacts' for binding and initiation of conformational changes. We anticipated that the badan modification (of the cysteine mutants) might restore the activity as the badan can substitute for the aromatic amino acids. However, the badan modification has not restored the hemolytic activity of α-HL, indicating the importance of proper penetration and conformational changes initiated by this amino acid segment. It has been argued that the W179 of Caveolin-1 binding motif can interact with pre-clustered head groups of sphingolipids in natural erythrocytes and artificial membranes, hence, might contribute for the clustering of α-HL at cell surface before penetration of its β-barrel [15–17]. This Caveolin-1 binding motif, as shown by us earlier, is structurally flexible, hence, can elicit differential mode of target binding, i.e., it can interact with Caveolin-1 of mammalian cells as well as phospholipids head groups like phosphotidyl choline [15]. Depending upon the target membrane viz. red blood cells, artificial membranes, and nucleated cells, this segment may exhibit the desired flexibility for membrane binding and penetration.

In summary we examined, nature of the Caveolin-1-binding motif and its relevance in membrane binding and β-barrel formation. We have been able to demonstrate that the intrinsically flexible Caveolin-1-binding motif co-operatively regulates assembly of α-HL.

Acknowledgments

The authors thank Prof. Surolia, Dr. Mishra, Dr. Islam Khan for valuable criticism, Mr. Anil Lotke for technical assistance. S.P. is recipient of senior research fellowship from CSIR. The financial assistance was provided by the Department of Biotechnology, Government of India.

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