Structural Characterization for N-Terminal Domain of Caveolin-1

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Caveolin-1 is a principal protein in the plasma membrane microdomains called caveolae. Caveolae play an important role in the transcytosis and pinocytosis. Therefore, caveolin-1 is most likely to work for the membrane dynamic events. In addition, caveolin-1 interacts with various signaling molecules. Although caveolin-1 possesses a variety of physiological functions, its structural properties were little construed. Here we analyzed the structural dynamics of the N-terminal caveolin-1 (residues 1-101), in order to better understand the structural properties in terms of its versatile functionality. We first analyzed its oligomeric form using GST-fused N-terminal domain, revealing that it equilibrates between a dimer and monomers in a concentration-dependent manner. The N-terminal domain of caveolin-1 was previously found to form a heptamer, so that our data suggest the dimeric form as an intermediate structure for the heptamer formation. Then, we obtained the folding profile, which indicated that ΔG_{H_2O} is about 0.5 ± 0.03 kcal/mol. The stability of N-terminal domain is relatively low, indicating that N-terminal domain may not be crystalline. Conclusively, the dynamic and flexible structure of N-terminal domain appears more favorable to maintain the versatile functions of caveolin-1.

Caveolin-1 is a principal protein of the cavela, a plasma membrane microdomain. Caveolae are found in most cell types including endothelial cells and smooth muscle cells (Scherer et al., 1997; Park et al., 1998). Endothelial cells contain a large population of caveolae that play important roles in vascular function including nitric oxide synthetase (NOS) regulation and blood flow responses (Park et al., 2000; Gonzales et al., 2002). Caveolae have been studied for decades and are more intensively studied recently since it was found to be involved in many diseases (Razani and Lisanti, 2001; Stan, 2002).

Caveolin-1 is a 22-kDa membrane protein that binds many different signaling molecules such as heterotrimeric G proteins, Src, Ras, and calmodulin (Li et al., 1995; Li et al., 1996; Song et al., 1996; Michel et al., 1997; Okamoto et al., 1998). Therefore it synergistically increases or inhibits some signaling pathways triggered by various stimuli including growth factors. There are two isoforms of caveolin-1, called caveolin-1α and β (Scherer et al., 1995). Caveolin-1α consists of 178 amino acid residues and caveolin-1β lacks the N-terminal residues (1-31). The localization of caveolin-1α and β seems different and their function appears distinct as well (Fujimoto,

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2000). The functional domains of caveolin-1 were unraveled as the oligomerization domain (residues 61-101), the scaffolding domain (residues 81-101) and transmembrane domain (residues 101-135) (Okamoto et al., 1998; Park et al., 2000). The transmembrane domain is hairpin-like, so that both N- and C-termini face the cytosolic side. It is of interest that the scaffolding domain was suggested to interact with many signaling molecules and inhibit their activities, indicating roles of caveolin-1 in many cellular events (Liu et al., 2002). To date a number of studies have been executed to determine a broad spectrum of caveolin-1 functions, yet the dynamics of caveolin-1 structure is only superficially elucidated. Although caveolin-1 forms an oligomer and interacts with many different signaling molecules, the structural mechanisms for its multiple binding propensity are not known.

Caveolin-1 forms homotypic oligomers of up to 18-mer. Recently, residues (1-101) were found to form a heptamer *in vitro* (Fernandez et al., 2002). The oligomerization may play an important role in construction of the caveolae coat. In addition, its oligomerization may be essential for compartmentalization of the signaling molecules. Therefore, the underlying mechanisms for the oligomerization must be uncovered to understand physiological or pathophysiological roles of caveolin-1.

The current works address the mechanisms for the caveolin-1 oligomerization, the stability of caveolin-1

oligomers and the structural diversity of caveolin-1, providing insight on its involvement in a variety of cellular functions.

Materials and Methods

Caveolin-1 expression and purification

Construction of GST-fusion proteins containing the full length or fragments of caveolin-1 and purification of the recombinant fusion proteins from *Escherichia coli* lysates by glutathione-agarose affinity chromatography were described previously (Scherer et al., 1995). In brief, the *E. coli* cells were induced by 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) and grown for 4 h at 37°C. Then, cells were harvested by centrifugation and lysed by sonication after incubating in the lysis buffer (150 mM NaCl, 7.5 mM Tris HCl, pH 8.0, 3 mM EDTA, 5 mM dithiothreitol and 1.5% N-lauryl sarcosyl) containing 100 μg/ml of lysozyme. Then insoluble cell debris was removed by centrifugation (15,000 rpm) and the GST-fused proteins were separated using glutathione agarose bead (Pharmacia Biotech). Purified proteins were resolved by 12.5% SDS-PAGE, analyzed by Coomassie brilliant blue staining and by Western blotting using a polyclonal anti-GST antibody (Santa Cruz).

Size exclusion chromatography

Purified GST-fused caveolin-1 deletion mutants were loaded to the Hiprep 26/60 Sephacryl S-200 HR column (Amersham-Pharmacia) pre-equilibrated with PBS and eluted by using FPLC (Amersham-Pharmacia Biotech). The size of each mutant was determined by comparison with the elution volume of standard proteins of known molecular size (Amersham-Pharmacia Biotech). Protein samples were eluted at a flow rate of 1 ml/min and the eluted proteins were detected by ultraviolet (UV) absorbance at 280 nm.

Equilibrium denaturation

Equilibrium denaturation as a function of urea concentration was monitored by UV differential spectroscopy using a spectrophotometer (Amersham-Pharmacia-Ultraspec 2100 Pro). Protein samples at indicated concentrations were equilibrated at various concentrations of urea at 20°C and the differential absorbance was measured. Nonlinear least-square fit to the equilibrium data was executed by using Origin software (provided by Microcal) and the following Santoro-Bolen equation (Santoro and Bolen, 1988):

$$X_D = \{(X_N + a_1[\text{Urea}]) + (X_U + a_2[\text{Urea}]) \exp[-(\Delta G_{H2O}/RT + m[\text{Urea}]/RT)]\}/\{1 + \exp[-(\Delta G_{H2O}/RT + m[\text{Urea}]/RT)]\}$$

where, X_N and X_U are the intercepts, and a_1 and a_2 are the slopes of the baselines at low and high urea concentrations, respectively. ΔG_{H2O} is the apparent free energy difference between the folded and unfolded forms of the protein linearly extrapolated to [Urea]=0, and m is the slope describing the dependence of ΔG_{H2O} on [Urea].

Reversibility

In order to refold proteins, urea was removed by dialysis against a refolding buffer (150 mM NaCl, 7.5 mM Tris-HCl, pH 8.0, and 3 mM EDTA) and then protein amount was measured by the Biorad assay (Bio-Rad Laboratories). Then, the absorption spectroscopy for refolded samples was obtained at the same concentration of unfolded samples. Functional reversibility was monitored by the recovery of the binding property of the refolded caveolin-1 (1-101) protein. Binding assay was determined by GST-pull down assay. Bead-binding GST-caveolin-1 (1-101) was obtained by incubation with excessive amount of GST-caveolin-1 (1-101) for more than 4 h at 4°C and washing at least four times with PBS. The 40 μl 1:1 slurry of bead-binding GST-caveolin-1 (1-101) was incubated with 50 μg each of thrombin-treated purified caveolin-1 (1-101), urea-treated samples or dialysis-performed samples for more than 4 h at 4°C. Then bead was washed thoroughly, boiled after mixing with Laemmli sample buffer, and resolved by 12.5% SDS-PAGE. Then the bound thrombin-treated purified caveolin-1 (1-101) was detected by Western blot using a polyclonal caveolin-1 antiboby (Santa Cruz).

Results

GST-caveolin-1 (1-101) forms a dimer

Previously it was elucidated that caveolin-1 forms homotypic oligomers *in vitro* and *in vivo* (Okamoto et al., 1998). The oligomerization of caveolin-1 has been focused to understand its roles in the signal transduction, because the caveolin-1 oligomer is considered as a center for some structural and functional regulation of the signal transduction. However, little was known about the exact stoichiometry and the dynamics for the caveolin-1 oligomerization. *In vitro* experiments using the full-length caveolin-1 are not easily performed due to its insolubility. Recently, caveolin-1 (1-101) was found to form a heptamer, determined by gel filtration and analytical ultracentrifuge (Fernandez et al., 2002). Thus, we intended to determine whether the heptameric form is stable when caveolin-1 interacts with other signaling molecules and whether it is also stable at very low concentration *in vivo*.

To understand the stability and dynamics of the caveolin-1 (1-101) heptamer, we examined the stoichiometry for oligomer of GST-caveolin-1 (1-101). First, we purified GST-caveolin-1 (1-101), GST-caveolin-1 (1-61) and GST-
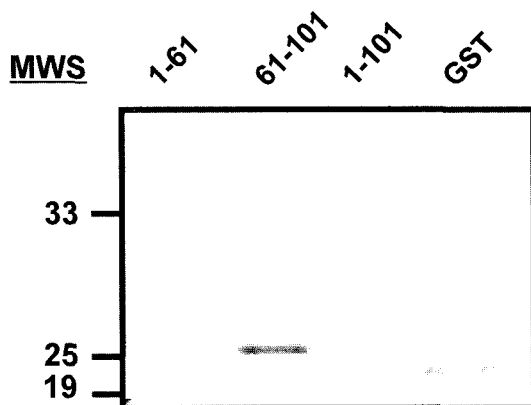
Fig. 1. SDS gel electrophoresis for purified GST-caveolin-1 mutant proteins. *GST-caveolin mutant constructs were purified as described in Materials and Methods. Purified proteins were resolved by SDS-PAGE and stained with Commassie Brilliant Blue. Numbers represents caveolin-1 residues (1-61: GST-caveolin-1 residues (1-61), 61-101: GST-caveolin-1 residues (61-101), 1-101: GST-caveolin-1 residues (1-101), GST: glutathione S-transferase control).*

caveolin-1 (61-101) (Fig. 1). Residues (61-101) were known as an oligomerization domain, referred to as an essential domain for homotypic oligomerization (Okamoto et al., 1998). Therefore, we constructed three different mutant structures. The oligomerization status for purified proteins was then analyzed by size-exclusion chromatography (Fig. 2). The GST-caveolin-1 (1-101) and GST-caveolin-1 (61-101) form dimers, indicating that caveolin-1 (1-101) and (61-101) maintain their binding properties. GST-fused proteins could lose their functional activity due to structural interruption of the additional GST domain, but GST-caveolin-1 (1-101) and GST-caveolin-1 (61-101) may not be the case, because of their functional maintenance.

Whereas GST-caveolin-1 (1-101) was dimerized at high concentration (3.4 mg/ml) (Fig. 2), its dimer was dissociated into monomer at 10-fold lower concentration (0.5 mg/ml). This result indicates that the caveolin-1 (1-101) construct is likely to be an intermediate dimer in the process of heptamerization. When the GST-caveolin-1 (1-101) protein was concentrated up to 6.0 mg/ml, it was still shown to be a dimer (data not in shown). Accordingly, the dimerization was concentration-dependent, implying that the structural form of GST-caveolin-1 (1-101) exists in dimer-monomer equilibrium. This further suggests that caveolin-1 oligomer can be dissociated at very low concentration in the plasma membrane, although caveolin-1 exists as oligomers in caveolae because caveolin-1 is locally concentrated.

Reversibility

To know the stability of the oligomer of caveolin-1 (1-101), we performed urea unfolding experiment. The caveolin-1 protein used in the unfolding studies was
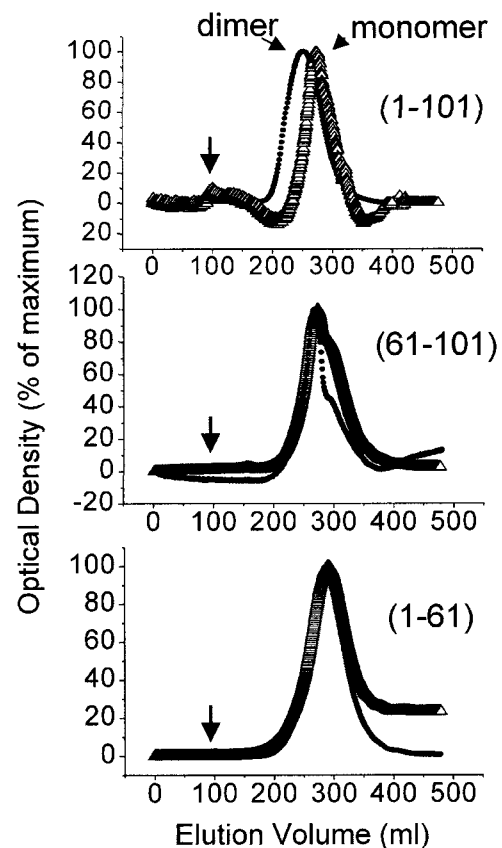

Fig. 2. The GST-caveolin-1 residues (1-101) exist in dimer-monomer equilibrium. *The purified GST-fused residues (1-101), (61-101) and (1-61) were analyzed by size-exclusion chromatography. Residues (1-101) form dimer at the concentration of 3.4 mg/ml, whereas they are determined as a monomer at the 0.5 mg/ml. Monomer and dimer of residues (61-101) existed concurrently at 1.5 mg/ml and 0.2 mg/ml. However, residues (1-61) were found as a monomeric form at 2.0 mg/ml and 0.1 mg/ml. Arrows are indicative of void volume.*

obtained as caveolin-1 (1-101) after GST-caveolin-1 (1-101) was cut with thrombin and GST protein was removed by the glutathione-bead binding (Fig. 3). Then, we tested whether the unfolding/folding process was reversible. The difference of optical density was increased when the caveolin-1 (1-101) was incubated in the unfolding buffer (8 M urea). Interestingly, the refolding protein after dialysis against refolding buffer had little difference in optical density of native and refolded samples (Fig. 4A). Furthermore, we also examined the reversible self-association of caveolin-1 (1-101) in the unfolding/folding process. As shown in Fig. 4B, the refolded caveolin-1 (1-101) protein was capable of associating with GST-caveolin-1 (1-101), implicating that caveolin-1 (1-101) was functionally reversible in the 8 M urea denaturation/renaturation.
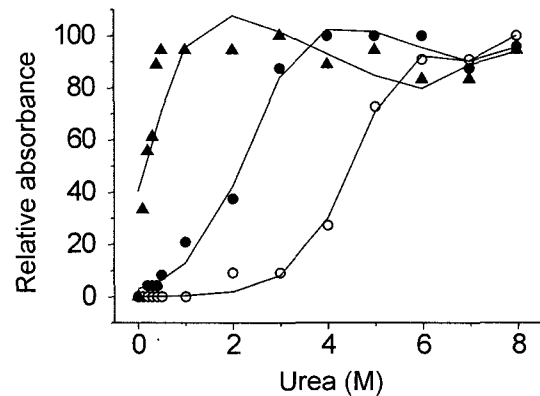
The stability of caveolin-1 (1-101)

The folding profile for the caveolin-1 (1-101) protein was
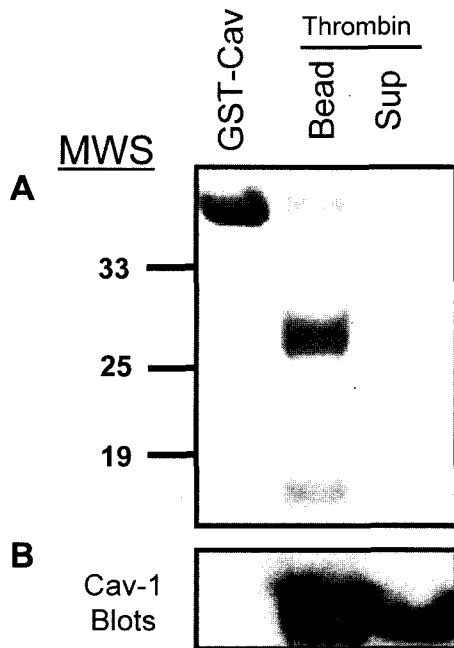
Fig. 3. Caveolin-1 residues (1-101) were obtained by the thrombin treatment. GST-fused residues (1-101) were digested by thrombin and the residues (1-101) were separated by glutathione-bead binding and subsequent centrifugation. Panel A shows proteins of each fraction resolved by SDS-PAGE (GST-Cav: control, bead: bead binding fraction after thrombin-treatment, sup: supernatant fraction after thrombin-treatment) and panel B shows Western blots developed by using polyclonal caveolin-1 antibodies.

Fig. 5. Folding profiles. The unfolded fraction was detected by difference in optical density at 275 nm. Closed triangles, closed circles and open circles represent data points at concentration of 0.01, 0.02 and 0.05 mg/ml, respectively. The data was fitted by the Santoro-Bolen equation. Two independent experiments were carried out and average values were plotted.

monitored by difference in optical density at various concentrations of urea (Fig. 5). Fitting was performed at all three different data sets by using the Santoro-Bolen

equation (see Materials and Methods). Interestingly, the folding profile was various at different concentrations of caveolin-1 (1-101). In other words, the folding profile was concentration-dependent, suggesting that the heptameric form was dissociated into smaller intermediate forms at lower concentrations. Therefore, in case of data obtained at high concentrations, non-linear square fitting may not be feasible with the simple two-state model (Folded ⇔ Unfolded), because dissociation of an oligomer makes it more complicated. However, the two-state model is possibly applicable to fit the data obtained at a very low concentration. At very low concentrations, caveolin-1 (1-101) may exist as a monomer that must be directly
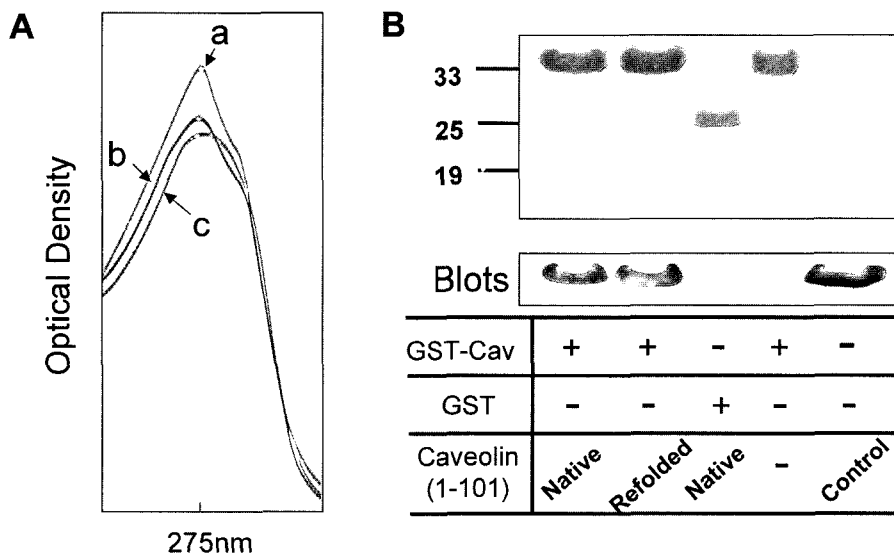
Fig. 4. Reversibility for the urea denaturation. Caveolin-1 residues (1-101) were denatured by 8 M urea-incubation for 18 h. Then the denatured sample was dialysed against the refolding buffer. In panel A, protein (0.02 mg/ml) was scanned by uv spectroscopy (a: the sample in 8M urea, b: the native sample, c: the renatured sample). In panel B, GST or GST-fused caveolin-1 residues (1-101) bound to glutathione-beads were incubated with purified residues (1-101), washed thoroughly and resolved by SDS-PAGE. Coomassie Brilliant Blue staining is shown in the upper panel and Western blots using polyclonal caveolin-1 antibodies are shown in the lower panel.

unfolded without dissociation. Therefore, we executed non-linear square fitting with data obtained at the lowest concentration (10 μg/ml), and the fitting generated ΔG_{H_2O} as 0.5±0.03 kcal/mol. We also obtained a folding profile at a 5-fold lower concentration by intrinsic fluorescence measurement, exhibiting UV spectral profile very similar to that obtained at the lowest concentration (data not shown). Of note, these folding profiles show as well that the stability of caveolin-1 increases as its concentration increases.

Discussion

Caveolin-1 was known to be oligomers *in vivo* and also work as scaffolding "Velcro" for various signaling molecules. So far has not been evaluated whether caveolin-1 is "stiff" or "flexible". In case of the full-length caveolin-1, it is hard to execute structural studies due to its insolubility. Recently, it was reported that caveolin-1 (1-101) forms a heptamer, and further suggested that it forms a bigger complex (Fernandez et al., 2002). On the other hand, the current works address the dynamic state of caveolin-1. First, caveolin-1 is likely to make a concentration-dependent association. Caveolin-1 forms oligomers at high concentrations, whereas the complex is possibly dissociated into smaller one at relatively low concentrations *in vivo*. Second, our data suggest the dimeric form as an intermediate structure for heptamer formation. Third, the stability of caveolin-1 decreases as its concentration becomes lower, implicating that the structure of N-terminal cytosolic domain is less crystalline at relatively lower concentrations.

The localization of caveolin-1 is, not limited, because it was found in various loci, from the plasma membrane to interior subcellular organelles (Liu et al., 2002). Additionally, the caveolin-1 protein moves dynamically between subcellular organelles and the cell surface and it also interacts with multiple cell-signaling molecules. To efficiently employ the versatile interactions with many different molecules and dynamic alteration in localization, the dynamic change in its conformations may be more reasonable, because this supplies caveolin-1 with abundant binding surfaces. Here, we obtained corresponding results suggesting that the three-dimensional structure of caveolin-1 is dynamically flexible, rather than forming a stiff crystalline. More confidentially, caveolae consisted of caveolin-1 are involved in transcytosis or pinocytosis, i.e., the dynamical movement of the plasma membrane (Stan, 2002). Given that caveolin-1 is intimately related with the transcytosis or pinocytosis, its structural diversity should be a pivotal concept for mechanistically understanding the functional-based structure of caveolin-1.

Acknowledgement

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