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Biophysical effect of lipid modification at palmitoylation site on the structure of Caveolin 3

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Abstract Caveolae are small plasma membrane invaginations that play many roles in signal transduction, endocytosis, mechanoprotection, lipid metabolism. The most important protein in caveolae is the integral membrane protein, caveolin, which is divided into three families such as caveolin 1, caveolin 2, and caveolin 3. Caveolin 1 and 3 are known to incorporate palmitate through linkage to three cysteine residues. Regulation of the protein palmitoylation cycle is important for the cellular processes such as intracellular localization of the target protein, membrane association, conformation, protein-protein interaction, and activity. However, the detailed aspect of individual palmitoylation has not been studied. In the present work, the role of each lipid modification at three cysteines was studied by NMR. Our results suggest that each lipid modification at the natively palmitoylation site has its own roles. For example, lipidations to C106 and C129 are play a role in structural stabilization, however, interestingly, lipid modification to C116 interrupts the structural stabilization.

Keywords Caveolae, Caveolin3, lipidation, NMR, transverse relaxation

Introduction

Caveolae, first identified by electron microscopy, are 50-100nm invaginations presented in the plasma membrane and they are ubiquitous in a wide range of mammalian cell type such as adipocytes, endothelial cells, fibroblasts, and smooth muscle cells.[1, 2] Caveolae enriched in cholesterol, glycosphingolipids, and lipid-anchored membrane proteins are involved in a wide variety of cellular processes including clathrin-independent endocytosis, cellular signaling, lipid metabolism and mechanosensation.[3-5]

Caveolins are the principal proteins of caveolae. The integral membrane protein family of caveolins has been the major biomarker of caveolae since their identification and the main topic of caveolae research.[6, 7] The caveolin family consists of Caveolin 1 (Cav1), Caveolin 2 (Cav2), and Caveolin 3 (Cav3). Cav1, together with Cav2, has been most extensively characterized among the caveolin family and is expressed in a various tissues including adipocytes, endothelia, and type-1 pneumocytes.[8, 9] On the other hand, Cav3 is predominantly expressed in skeletal and smooth muscle tissue.[10] Cav1 and Cav3 have pivotal roles in the formation of caveolae; genetic ablation of caveolin cause loss of caveolae, and Cav1 in cells lacking endogenous caveolae is able to produce *de novo* formation of caveolae.[6, 11, 12] Furthermore, some

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amino acid substitutions in Cav3 were known to be related to diseases such as limb-girdle muscular dystrophy (LGMD-1C), duchenne muscular dystrophy, asymptomatic hyperCKemia, rippling muscle disease (RMD).[10, 13, 14] Thus, investigation of biophysical characteristics of Cav3 is important for understanding functional roles and its relations to human disease.

Cav3 consists of 151 amino acids and is generally divided into four structural domains: a flexible N-terminal domain (residues 1-54), a scaffolding domain (res. 55-74), a membrane-embedded domain (75-106), and a C-terminal cytosolic domain (res. 107-151).[4] The N-terminal domain contains a signature motif (FEDVIAEP) which is highly conserved among caveolin family. However, its functional roles are unclear yet. The scaffolding domain has been reported to bind various proteins and plays a key role in homo-oligomerization of which the detailed mechanism has not been clearly elucidated.[15, 16] The membrane-embedded domain exhibits a helix-break-helix structure that penetrates deeply into membrane bilayers.[17] C-terminal domain composed of several helices possesses three cysteines which are subject to palmitoylation.[18]

Protein palmitoylation is a post-translational and generally reversible modification that is distinguished from protein myristoylation, which is cotranslational and blocked by protein synthesis inhibitors.[19] Protein palmitoylation and depalmitoylation reactions are thought to be primarily enzymatic and catalyzed by palmitoyl acyltransferases and by palmitoyl thioesterases, respectively.[20, 21] Regulation of the protein palmitoylation and depalmitoylation cycle play important roles in intracellular localization of the target protein, membrane association, conformation, protein-protein interaction, and activity. Three cysteine residues (residues 133, 143, and 156) on Cav1 are known to undergo irreversible palmitoylation that likely impacts on caveolin oligomerization, lipid-membrane interactions, and function.[18, 22] These cysteines are conserved in Cav3 (residues 106, 116, 129), which is also known to be palmitoylated,[23] however the detailed pattern of palmitoylation has yet to be determined. In the previous work, lipid modification to the three natively palmitoylated residues was proved to exert a modest and local effect on the Cav3 structure.[24] However, the biophysical effect of individual lipidation to palmitoylated residues has not yet been determined. In this study, we characterized biophysical behavior of artificial lipidation to the individual cysteines.

Experimental Methods

Cloning and Plasmid Construction The cDNA of human Cav-3 was amplified by polymerase chain reaction (PCR), and the amplified cDNA was cloned into a pET16b vector with an added segment encoding N-terminal poly histidine to facilitate purification.

Expression and purification of Cav3 Harvested cells were suspended in 20ml lysis buffer (75 mM Tris, pH 7.8, 300 mM NaCl) per gram of wet cells. The lysis buffer contained 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM magnesium acetate, 2 mg/mL lysozyme, and 0.2ml of phenylmethylsulfonyl fluoride (PMSF) per gram of cells. The mixture was tumbled for 90 min at room temperature followed by sonication on ice. Inclusion bodies pelleted by centrifugation at 20,000 rpm for 30 min were solubilized in 20 mL of buffer A (40 mM HEPES pH 7.8, 300 mM NaCl) containing 3% (v/v) N,N-Dimethyl-N-dodecylglycine betaine (Empigen, Sigma-Aldrich) detergent. The solution was then tumbled at 4°C for 2 hours, and remained insoluble particles were removed by centrifugation. Clarified soluble fraction were loaded into Ni-NTA resin column and sequentially washed with buffer A containing 3% (v/v) Empigen and buffer A containing 40 mM imidazole and 1.5% (v/v) Empigen, which eluted non-specific His6 binding impurities from the resin. Empigen was then exchanged for 0.1% lyso-palmitoylphosphatidylglycerol (LPPG) by re-equilibrating the column with 20 column volumes of 20 mM sodium phosphate (pH 7.2) containing detergent. Cav3 was then eluted from the column with 250 mM imidazole (pH 7.8) and 1 mM DTT.

Mutagenesis and lipidation of Cav3 The mutant form of Cav3 was employed that six of the native cysteine sites were mutated to Ala or Ser (C19S, C72A, C94A, C98A, C124S, and C140S). Mutant variants were generated by site-directed mutagenesis using the

QuikChange site-directed mutagenesis kit. The three Cys sites (C106, C116, and C129) of which are known to undergo palmitoylation were either lipidated or mutated to Phe. For the studies of this work, lipidation was carried out via linkage of a thiooctyl groups to the Cys sites through a disulfide bond. The detailed methods of lipidation to cysteine residue were previously described.[24] The final concentration of protein was determined by measuring the A280 and using an extinction coefficient of 30940 $M^{-1}cm^{-1}$.

NMR Spectroscopy 2D ^{1}H-^{15}N TROSY NMR spectra [25] were acquired using Bruker 900 MHz spectrometers equipped with TXI cryoprobes. T2 values were measured from ^{1}H-^{15}N correlation spectra recorded using relaxation evolution delays of 16, 32, 48, 64, 80, 112, 144 and 192 ms. A delay of 3.5 s was used between scans. NMR data were processed with the NMRPIPE[26] and analyzed using the SPARKY program.[27]

Results and Discussion

^{1}H-^{15}N TORSY-HSQC NMR spectra comparison of 568C, 5F68C, 6F58C, and 8F56C-Cav3 Monomeric Cav3 was thought to be composed of an unusual and mainly flexible structure for which NMR is a proper way to study. In the previous work, LPPG was selected as an optimal detergent for NMR studies of Cav3 based on ^{1}H-^{15}N TROSY spectrum screening with various detergent, and artificial lipidation to three native palmitoylation sites was also conducted to enhance the quality of NMR spectrum.[24] However, biophysical effect of hydrophobicity enhancement at individual palmitoylation sites on the structure of Cav3 was unclear. To elucidate this issue, we replaced a natively palmitoylated cysteine residue in Cav3 with phenylalanine, which increases hydrophobicity at the mutated site. The other cysteine residues unrelated to palmitoylation were replaced with alanine or serine as described in previous work. The construct in which the

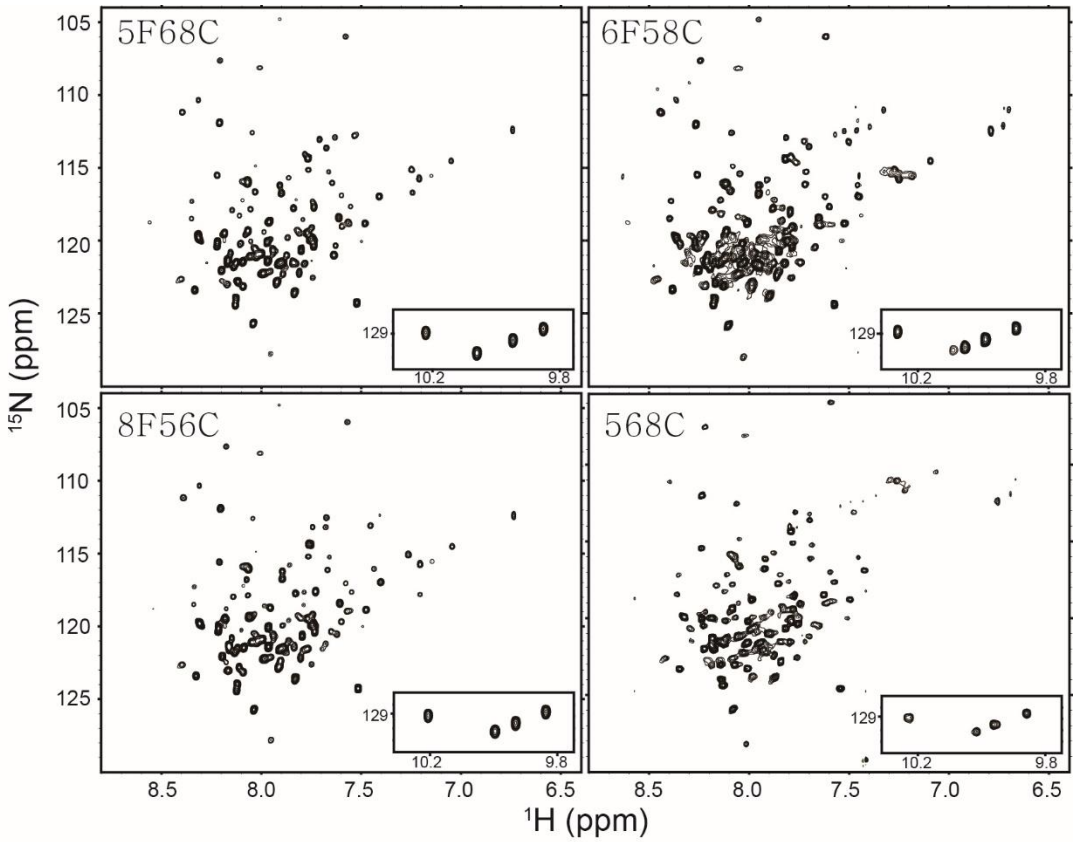

Figure 1. Comparison of the 900 MHz ^{1}H-^{15}N-TROSY spectra of full-length mutated Cav3 in LPPG detergent micelles (1 mM EDTA, 1 mM DTT and 100 mM imidazole, pH 6.5, and 318K). The inset panel shows the tryptophan indole peaks. The expected number of tryptophan side chain peaks is 4.

fifth cysteine residue from the N-terminus that is the first palmitoylated site was mutated to phenylalanine (C106F) while maintaining the other two lapidated cysteines (C116 and C129, 6th and 8th cysteines, respectively) is termed 5F68C-Cav3. In this way, 568C-Cav3, 6F58C-Cav3 and 8F56C-Cav3 were also produced.

The backbone amide resonances of 568C-Cav3 in the ^{1}H-^{15}N TROSY spectrum exhibit narrow dispersion and line broadening of some peaks, suggesting that the protein is composed of disordered and/or α-helical structure. The NMR line-width is directly proportional to observed transverse relaxation rate $R_{2,obs} = R_2 + R_{ex}$ indicating the rate at which the X (or Y) component of the magnetization vector decays over time.[28] The presence of chemical exchange cause dephasing the precessing frequency, which leads to an additional exchange contribution referred as R_{ex}. It was hypothesized that the increased hydrophobicity through phenylalanine substitutions instead of palmitoylation at the natively palmitoylated site can also suppress the dynamic behavior by making the C-terminus region stick to the membrane environment, resulting in a ^{1}H-^{15}N TROSY spectrum with less line-broadening. As a result, the ^{1}H-^{15}N TROSY spectrum of 5F68C showed better quality in terms of the line-broadening and peak number than other constructs, suggesting that first palmitoylation may be important for stabilizing C-terminus region, which is in concordance with the fact that C133 residue in Cav1 corresponding to C106 in Cav3 is known to be the predominant site of palmitoylation. NMR spectrum of

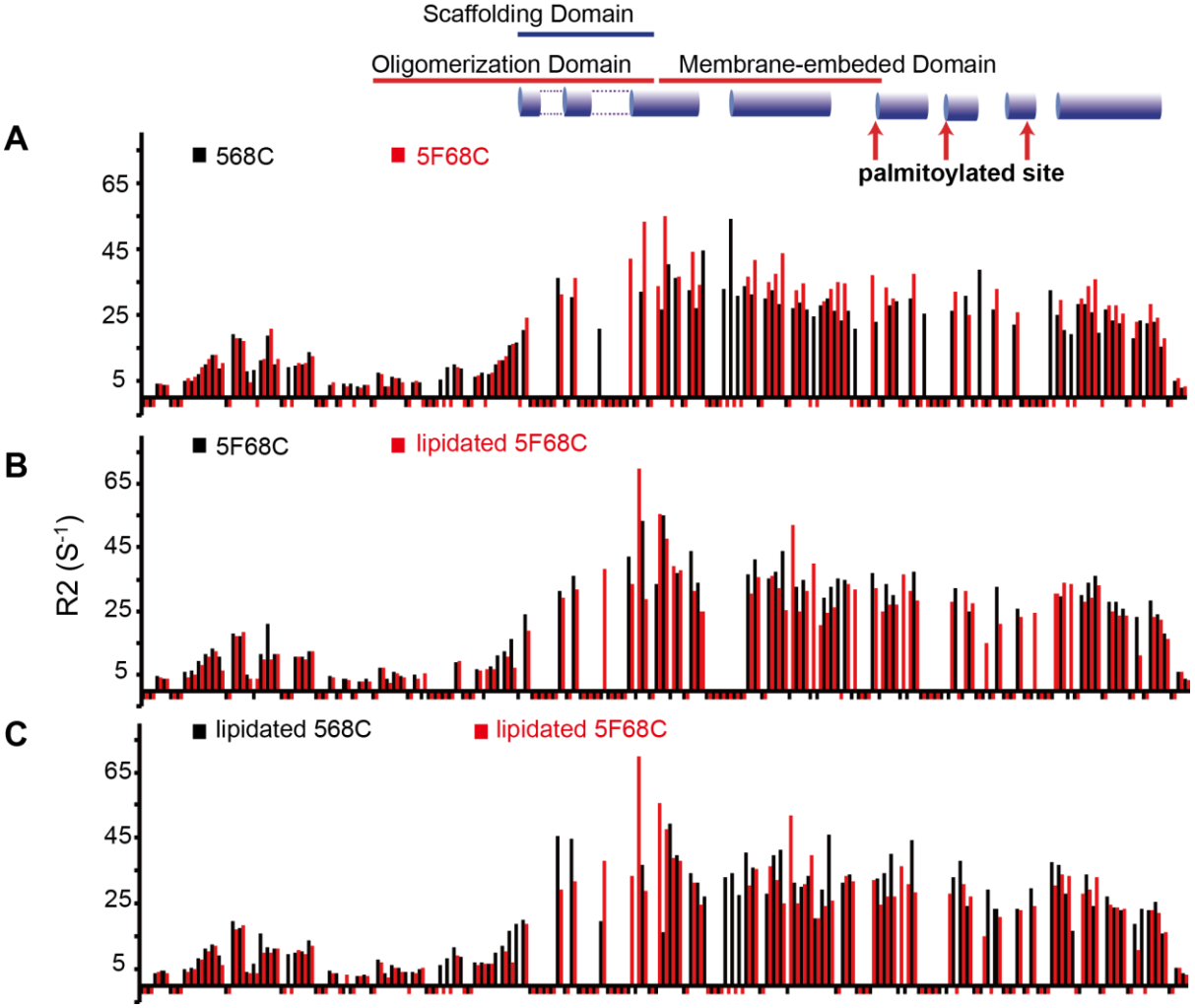

Figure 2. Transverse relaxation rates (*R2*) comparisons of 568C-Cav3, lipidated 568C-Cav3, 5F68C-Cav3, lipidated-5F68C. Negative values indicate residues that could not be assigned due to peak broadening or overlap.

8F56C-Cav3 also show the promising resonance peaks, while severe line-broadening was observed in the spectrum of 6F58C-Cav3. These results suggest that all palmitoylation at the three cysteines don't suppress protein dynamic behavior.

Dynamic analysis of lipidated or non lipidated 568C and 5F68C-Cav3 In order to confirm the effect of the palmitoylation on the structure, we artificially lipidated to natively palmitoylation site and measured *R2* values which contain information about spin-spin relaxation and magnetic inhomogeneity. The palmitoylation was hypothesized to suppress the magnetic inhomogeneity caused by extra exchanges. As shown in the figure 2A, *R2* comparison between 568C-Cav3 and 5F68C-Cav3 shows that phenylalanine substitution increases the *R2* values of the residues in the membrane domain and C-terminal domain, which implies the rigidity of this region is increased, as a result of anchoring of this region to the micelle surface. The comparison between 5F68C-Cav3 and lipidated 5F68C-Cav3 showed interesting result in which lipidations on the protein decreases the *R2* values than non-lipidated protein (Fig. 2B). However, three lipidations at the palmitoylation sites increase R2 values comparing to the two lipidations at the C116 and C129 residues (Fig. 2C). These results suggest several points. First, it is confirmed that palmitoylation at C106 is important and efficient position for structural stabilization. Second, lipid modification at C116 may interrupt protein stabilization. The functional role of the palmitoylation at C116 has not been reported yet, but it is expected that there will be a role other than structural stability. Third, the palmitoylation at C129 plays roles in structural stabilization as well as interactions with other proteins, which is reported previously.[29] The biophysical results in the present work suggests that each lipid modification at the natively palmitoylation site has its own roles, i.e. lipidation to C103 for structural stabilization and C129 for interaction with other proteins, which were not seriously discussed before.

As mentioned above, observed *R2* is a complex value composed of spin-spin relaxation and magnetic inhomogeneity, therefore, dynamic analysis based on measured *R2* has limitation that the two components cannot be distinguished directly. There are some ways to measure R_{ex} directly such as *R2* relaxation dispersion and R1ρ relaxation dispersion, however, those measurements are often very challenging for membrane proteins. This present work providing a preliminary information of protein dynamics through simple experiments would contribute understanding about the role of each palmitoylation on the structure of caveolin.

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