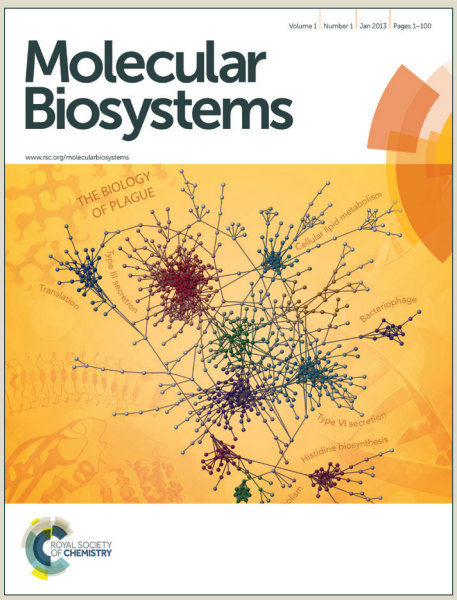
Molecular BioSystems

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

The Critical Residues of Helix 5 for *in vitro* Pentamer Formation and Stability of Papillomavirus Capsid Protein, L1

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

The mono-site mutations of the absolutely conserved residues, ^{464}LGR^{466}, in the α-helix 5 (h5) of HPV16 L1 completely disrupted the pentamer formation. The implication of this finding is the potential usage of a h5-like peptide as the reagent to interfere with the pentamer formation and stability as an anti-HPV reagent.

Papillomaviruses (PV) are nonenveloped viruses that infect epithelial tissues, inducing warts and other benign or malignant lesions. Over 100 different types of human papillomavirus (HPV) have been identified to date.[1] Cervical carcinoma, the second most frequent cancer in women worldwide is strongly associated with a preceding, high-risk HPV genital infection.[1,2] HPV vaccines that contain virus-like particles (VLPs) of the L1 capsid protein from several HPV types have proven to be effective against HPV infections.[3] Currently, two HPV VLP vaccines are available on the market.[3,4] These vaccines can effectively prevent HPV infection and the associated cervical cancer.

The L1 protein contains all the information needed for viral particle assembly. The *in vitro* assembly of L1 occurs spontaneously under high salt and low pH conditions. The size and quality of the assembled particles can be regulated by N-terminal[5] and C-terminal truncations.[6] Previous studies have primarily focused on VLPs assembly from the L1 pentamers, which can be purified as a stable unit. Only rare studies have focused on the formation of L1 pentamers from monomers.[6b,7] The high resolution, T=1, small VLP assembly of HPV16 L1 exhibited an atomic structure of twelve L1 pentamers in the particle, and the pentamer-pentamer interactions were essential for particle assembly.[6a] Furthermore, the truncation study on the L1 pentamer[7b] demonstrated that, near the C-terminus, the α-helix 2 (h2) and h3 were essential for L1 folding and producing the pentameric L1, and that the h4 was indispensable for particle assembly. Moreover, in deletion studies, a small helix at the C-terminus, h5, was also shown to be critical for L1 folding.[6] However, the exact role of individual residues of the short h5 in L1 folding and pentamer formation remains unclear.

An incidental mutation of R466 in the h5 of HPV16 L1 to Ala (R466A) produced only monomeric peaks without pentameric peak (Fig.1), indicating its crucial importance in pentamers formation. Such a finding is significant as it stopped the life cycle of virus at the pentamer stage, the prerequisite of virus particle assembly, by performing only mono-site mutation (R466A). To understand the structural role of h5, we further mutated each of the highly conserved residues, ^{464}LGRKFL^{469}, in the h5 of HPV16 L1 to Ala, and examined mutant proteins solubility, stability, and ability to form pentamers.

We performed mono-site alanine scanning mutagenesis of the ^{464}LGRKFL^{469} sequence in the h5, based on the wild-type HPV16 L1 (16WT) and its GST fusion protein, GST-L1.[6] The GST-L1 proteins were purified from cell lysates with GST affinity chromatography and size-exclusion chromatography (SEC) (See the details in ESI). In the FPLC elution profile, 16WT showed two inclusion peaks in the target protein with elution positions at about 265 and 53 kDa (black line in Fig. 1), these positions corresponded to the molecular weights of the L1 pentamer (L1-P, major peak) and the L1 monomer (L1-M, very minor peak), respectively. The identity of the L1 protein at the pentameric and monomeric peak positions were confirmed by SDS-PAGE (Fig. S1) and Western blot analysis (top panel of Fig. 1). Part of the protein in the sample was eluted in the void volume as a large aggregate. The molecular weight of which was beyond the resolution of the size-exclusion column. A recent study[7a] showed that the proteins from the monomer peak could not assemble into pentamers, possibly because they existed as partially misfolded, metastable proteins.

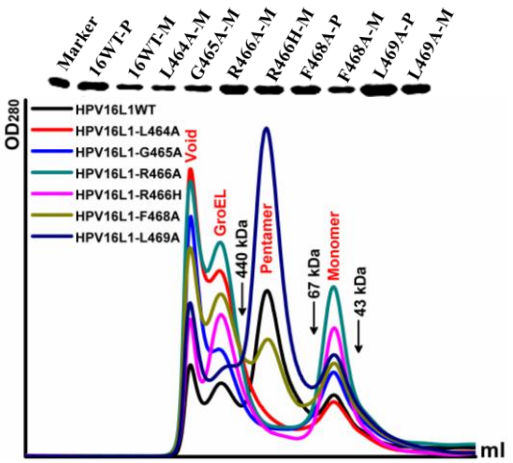

Fig. 1 The FPLC elution profiles of wild-type HPV16 L1 (16WT) and the HPV16 L1-h5 mutants. The positions of the molecular standards are indicated with arrows. A western blot (top) probed with a HPV16 L1 specific antibody verified the L1 identity of the proteins in the eluted monomer (M) and pentamer (P) peaks.

Among the mono-site Ala mutants, F468A and L469A also displayed two protein elution peaks that corresponded to L1-P and L1-M; however, the relative ratios of the peak heights were different from that of 16WT. In addition, L469A produced a higher yield of the L1-P protein, approximately two-fold higher than that of 16WT (Fig. 1). The higher solubility of L469A could be rationalized by the replacement of the highly hydrophobic Leu residue (which exists as five total Leu residues in the pentamer) with an Ala on the solvent-exposed surface of h5. The hydrophilic Ala enhanced the solubility of the already stable pentameric L1. Surprisingly, three mono-site mutants, L464A, G465A, and R466A, produced no pentameric peak, but they did produce monomeric peaks, these data indicated that these three residues were critical for the pentamer formation.

Next, L1-M sizes were determined by DLS (Fig. S2), which were consistent with those previously reported for L1-M[7a]. Afterwards, we compared the secondary structure and thermal stability of 16WT and the mutants. Circular dichroism (CD) spectra and UV cloud point-temperature (UV CP-Temp) analyses were performed with the soluble L1 proteins, obtained from the elution peaks of the SEC. The far-UV CD spectra of L1-P (Fig. S3A) and L1-M (Fig. S3B) proteins were respectively identical for F468A, L469A, and 16WT. These results indicated that these mutations did not induce major changes in the overall secondary structures of L1. However, quantitative analysis of the CD spectra for mutants L464A, G465A, and R466A (Table S1) revealed that the structures of these monomeric proteins (Fig. S3C) were somewhat different from those of L1-P and L1-M of 16WT (Fig. S3D). The UV CP-Temp experimental traces (Fig. 2) and the UV CP-Temp transition values (Table S2) revealed the thermal stabilities of these mutants. 16WT exhibited T_m values of 38.7 °C for L1-M and 53.2 °C for L1-P. These values indicated that the thermal stability of monomers were much lower than that of pentamers. Surprisingly, G465A and R466A mutants displayed a two-phase transition mode that was not observed in 16WT, which indicated clear differences in structural stability. This could suggest that, although each of these mutants showed only monomeric peaks in the FPLC elution profiles, the monomeric peak might represent a mixture of two different monomers, one with a structure similar to the 16WT monomer and the other with a different conformation induced by the mutation. To substantiate this hypothesis, an additional experiment was performed with heat treatment. A 10-min incubation at 47 °C induced aggregation and precipitation of the less stable form of L1, but the more stable form of L1 remained in solution. After centrifugation, the more stable, soluble L1 in solution was submitted to a UV CP-Temp measurement (Fig. S4) and CD spectra (Fig. S5). The data firmly supported the hypothesis that the monomeric peak of exhibited by the mutants (G465A and R466A) contained two conformations with different structural stabilities.

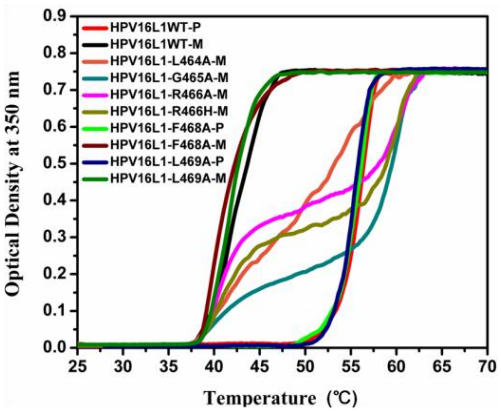

Fig. 2 The thermal stability of 16WT and HPV16 L1-h5 mutants were determined by UV cloud point with temperature ramping.

The R466A mutant behaved differently from the other mutants. It produced approximately 3-5 times more soluble monomeric L1 than any other Ala mutant, and it did not produce any pentameric L1, as shown by SEC and western blot analysis (Fig. 1). This unusually high yield of soluble monomeric L1 for R466A was surprising, because monomeric L1 generally has low solubility, as observed for 16WT and the other mutants (Fig. 1). To test whether the same behavior would be observed by mono-site substituting R466 with a polar residue, instead of the non-polar Ala, we mutated R466 in HPV16 L1 to His (R466H). In the FPLC elution profile (magenta line in Fig. 1), only the monomer peak was observed, confirming that the mutation of R466 led to soluble monomeric L1 protein. These results indicated that R466 indeed played a critical role in the pentamers formation. The far-UV CD spectra of R466H fell between those of L1-P and L1-M of 16WT (Fig. S3D). The UV CP-Temp of R466H (Fig. 2) displayed a two-phase transition process with T_m values of 38.3 and 52.9 °C (Table S2), essentially being identical to those of R466A, but largely different from those of 16WT. These results indicated that the intrinsic structure and stability of R466H/A were different from those of 16WT. To probe the general applicability of finding that the Arg residue in h5 was important for L1-P formation, we mutated the equivalent Arg in the wild-type HPV18 L1 (18WT) to His (R467H). HPV18 is another high-risk HPV associated with genital carcinoma.[8] The result (Fig. S6) demonstrated that the HPV18 R467H mutant was similar to the HPV16 R466H, in that no pentameric L1 was formed, but some soluble monomeric L1 was detected.

To understand further the functional and structural roles of h5 for L1 solubility, stability, and pentamer formation, we inspected in detail the structure of HPV16 L1 in conjunction with our mutational biochemical and biophysical data. We examined the h5 sequences in all papillomavirus types, including 32 different representative sequences from human and other species (Fig. S7). It indicates that the ^{464}LGRKFL^{469} sequence is highly conserved, and the ^{464}LGR^{466} sequence is absolutely conserved in L1 proteins of all papillomavirus types. This observation suggested that the ^{464}LGRKFL^{469} sequence in h5 must be important for L1 structure and function.

A detailed inspection of the crystal structure of the HPV16 L1 pentamer[6a] showed that the h5 was located inside the pentameric cavity, and it was anchored to the central β-barrel core of the subunit (Fig. 3A, B). In the context of a pentamer, the long side chain of R466 on h5 extends into a small groove of a neighboring subunit; this forms four hydrogen bonds with the neighboring subunit, one with V238, on the main-chain, and three with S239, E240, and Q317 residues on side-chains (Fig. 3C). These four hydrogen bonds are repeated five times in the pentamer, providing significant bonding interactions for maintaining the pentamer formation. Thus, besides stabilizing the monomeric subunit structure, this data provides another structural basis for

the important role of h5 in the pentamer formation. In particular, the residue R466 on h5 provides direct bonding interactions for the pentamer formation.

Because h5 plays a role in inter-subunit interactions, any mutations that affect its anchoring or folding would affect L1 stability and pentamer formation. The hydrophobic residues, L464, F468, and L459 formed intra-molecular hydrophobic packing through non-polar interactions with the residues of Y34, W313, and I375 in the β-barrel core (Fig. 3D). These interactions are very important for stable h5 anchoring to the β-barrel core of the same subunit (Fig. 3B), and thus, for L1 stability and pentamer formation. This structural feature provides a molecular explanation for the observation that L464A and F468A mutants produced greatly reduced soluble L1 pentamers and monomers, because the mutations destabilized h5 anchoring interactions on the β-barrel core. In addition, it is worth noting that mutation of another basic residue near R466 in 16WT, K467A, did not result in a failure to form L1-P proteins (Fig. S8). Inspection of the crystal structure of HPV16 L1 pentamer[6a,7c] indicated that K467 is on the exposed surface of h5, and it does not form any bonding contacts with the nearby subunit. Thus, K467 residue appears to be dispensable for L1 stability and pentamer formation.

Because stable anchoring of h5 on the β-barrel core structure is so important for the formation of L1 pentamer, these interactions may be targeted for inhibiting pentamer formation, and thus, inhibiting viral particle assembly. This notion is similar to the strategy of mediating or disrupting protein-protein interactions[9] in inhibitor and/or drug discovery. With this in mind, we created a 15-amino acid synthetic peptide that contained the h5 sequence and incubated it with 16WT protein to determine whether this h5 peptide (h5-pep) might inhibit pentamer formation. As shown in Fig. S9, the h5-pep effectively inhibited the pentamer formation of 16WT. This result suggested that the highly conserved h5 interaction site among papillomaviruses may serve as a target in developing potential new anti-HPV therapeutics.[10]

In conclusion, through site directed mutagenesis, we discovered that the mono-site mutations of the absolutely conserved residues, ^{464}LGR^{466}, in the h5 of HPV16 L1 completely disrupted the pentamer formation. Such a finding is significant because it halted one of the important steps of virus life cycle at the pentamer stage and therefore supplied a potential target for the development of anti-HPV reagents. In addition, a synthetic peptide including h5 effectively inhibited the pentamer formation of HPV16 L1. The third interesting finding was that that mutating a Leu residue to Ala on the solvent-exposed surface of h5 (L469A) produced an approximately two-fold pentameric yield of the wild-type HPV16 L1, being crucially important in lowering the cost of future HPV VLPs vaccine.

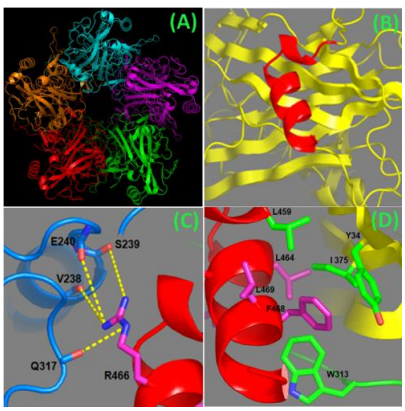

Fig. 3 Structure of the HPV16 L1 pentamer and inter-subunit interfaces (Protein Data Bank code 1DZL). (A) An illustration of L1 pentamer structure shows the five-fold axis, each monomer is shown in a different color. (B-D) The h5 is shown in red. (B) The h5 anchors to the central β-barrel core (in yellow) of the same subunit. (C) Ionic and hydrogen interactions occur between two monomers in a pentamer, R466 on h5 makes multiple bonding interactions with a neighboring subunit (in blue). (D) Hydrophobic interactions occur between h5 and the β-barrel core (in yellow) within the L1 monomer.

Acknowledgment: The present work was supported by the Natural Science Foundation of China (Nos. 91027027 and 20934002) and the State Key Laboratory for Supramolecular Structure and Materials, Jilin University.

Notes and references

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† Electronic Supplementary Information (ESI) available: [details of experimental procedures, DLS, CD spectra, FPLC, UV CP-Temp curves were presented as Fig. S1–S9]. See DOI: 10.1039/b000000x/

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