Crystallographically identical virus capsids display different properties in solution

Proteolysis and chemical modification experiments were combined with mass spectrometry to investigate viral capsid structural mobility in solution. Protein mass mapping was used to examine protein–RNA interactions and their influence on capsid mobility. Virus-like particles (VLPs) assembled in a baculovirus expression system and containing cellular RNA were compared with authentic virions containing the viral genome. Although the protein capsids were indistinguishable by crystallography, the VLPs were significantly more susceptible to cleavage of coat protein than virions. Results from site directed chemical modification of capsid proteins were also consistent with the proteolysis results. Particles containing cellular RNA were significantly more susceptible to modification. These observations demonstrate that lattice packing forces may mask differences in protein assemblies that are readily detectable in solution and that RNA regulates protein stability in a virus assembly.

The complexity of the viral life cycle, which includes particle assembly, transport to susceptible hosts, host selection and infection, requires that the capsid proteins perform a diverse set of functions with a minimum amount of genetic information. High resolution X-ray analysis of viruses has yielded detailed images of viral capsids in the crystalline state with structural implication for functions such as receptor binding and RNA release. The static nature of these studies requires additional experiments to understand mobility. A very promising recent approach is the use of limited proteolysis combined with peptide mass mapping[1,2]. It has provided evidence for the dynamic nature of viral capsids and has related capsid mobility to viral infectivity by detecting the exposure of normally internal structures in protease accessible environments

Flock house virus (FHV) is a well-characterized member of the RNA nodavirus family[3,4] and is an excellent system for investigating factors that affect viral assembly, stability and infectivity. The FHV capsid is composed of 180 copies of a 407 amino acid protein which undergoes an assembly-dependent auto-catalytic cleavage, resulting in a 44 amino acid peptide (γ) and a 363 amino acid major protein (β). FHV can be propagated in *Drosophila* cells, which give rise to infectious virions. Alternatively, the viral coat protein can be synthesized in a baculovirus expression system[5] which produces virus-like particles (VLPs) that are morphologically indistinguishable from authentic FHV except that they contain cellular RNA instead of the viral genomic RNA. Crystallographic comparisons of authentic FHV particles and VLPs show that they are indistinguishable at 2.8 Å resolution following refinement. Experiments comparing the rates of trypsin proteolysis of these particles showed unanticipated, substantial differences (Fig. 1) that implied variations in

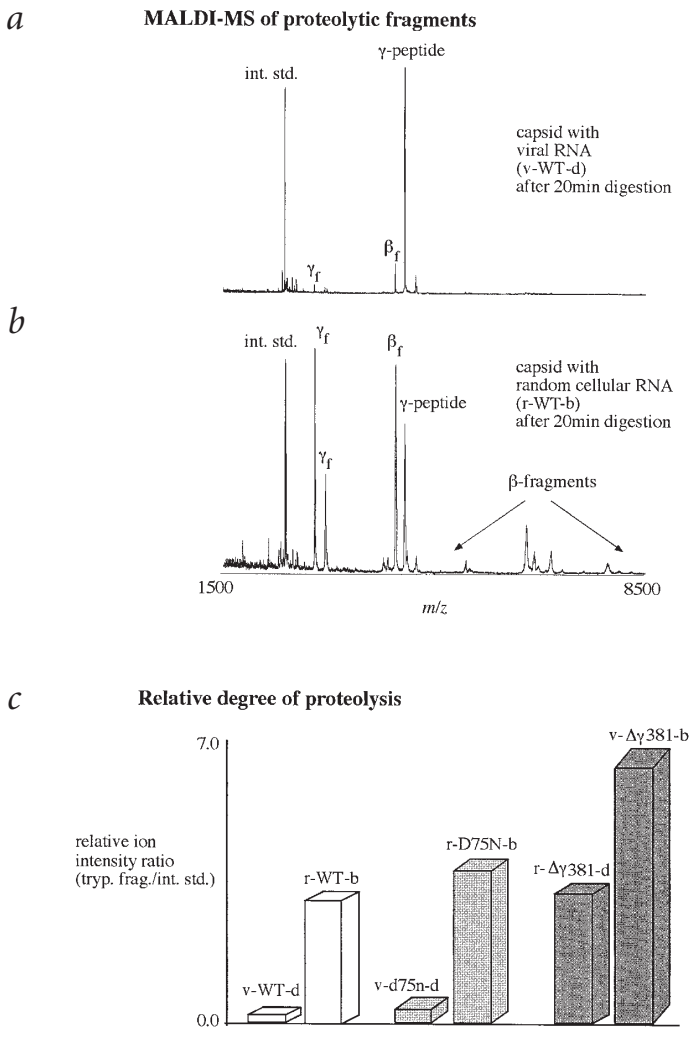

Fig. 1 MALDI-TOF mass spectra of tryptic peptides released from FHV and FHV VLPs. ***a***, v-WT-d particles containing viral RNA; ***b***, r-WT-b particles containing cellular RNA. γ_f: tryptic peptides from the cleavage of γ-peptide. β_f: tryptic peptides from cleavage of the major coat protein β. int. std.: a synthetic version of the γ-peptide consisting of the N-terminal 21 amino acids (mw 2,242 Da). A dramatic difference in the rate of digestion between particles packaging viral *versus* cellular RNA is observed based on the higher concentration (17-fold) of tryptic fragments seen in (*b*). The γ-peptide is a 44 amino acid peptide (mw 4,396 Da) resulting from the autocatalytic cleavage of the coat precursor protein. ***c***, A bar graph showing the influence of RNA on capsid dynamics: FHV particles containing viral RNA exhibit less dynamic motion than particles containing random cellular RNA. The rate of proteolysis is related to enzyme accessibility and is therefore a function of capsid dynamics. Particle types v-WT-d and v-D75N-d, the only particles that contain viral RNA, are significantly more stable than all other types tested.

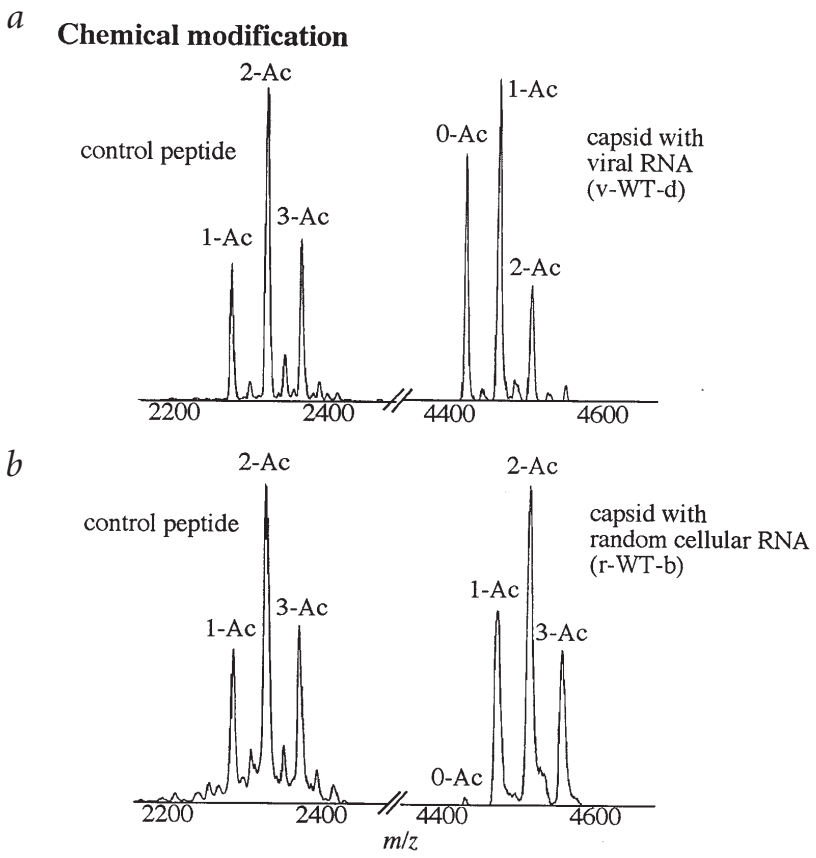

Fig. 2 Mass spectra of amino-acetylation reactions comparing reactivities of v-WT-d *vs* r-WT-b γ-peptide. ***a***,***b*** Spectra showing that sites on the v-WT-d γ-peptide (*a*) are less reactive than on the r-WT-b γ-peptide (*b*). Acetylation sites on the two different Δγ381 virions showed no difference in reactivity (data not shown). The results are consistent with the proteolysis experiments that demonstrated a dramatic difference in capsid dynamics depending on the type of RNA present inside the particle. Each reaction with acetic anhydride increases the peptide mass by 42 Da. Synthetic γ-peptide was added as an internal reference of reaction rate. Each γ-peptide (synthetic and particle associated) has three potential reactive sites.

their stabilities. The experiments described here were designed to explore the factors that cause such differences.

To investigate the nature of the observed variation in capsid protein dynamics, three sets of nucleoprotein particles were created using *Drosophila* cells and the baculovirus expression system (Table 1). Specifically, six types of particles composed of either wild-type or mutant FHV coat protein, were tested to elucidate the nature of the different capsid dynamics. One of the mutants contained a single amino acid change at position 75 (Asp to Asn), which created a cleavage-defective capsid[6]. The maturation cleavage event makes the particle resistant to disassembly by SDS[7] and the mutant was examined to see if this change in stability was accompanied by a change in the structural mobility of the capsid. The second mutant, Δγ381, was a C-terminal deletion of 26 amino acids, a region of the γ-peptide that is involved in recognition of viral RNA for assembly[8]. The Δγ381 coat protein packages mostly cellular RNAs even when synthesized in the native *Drosophila* system. Comparison of the *Drosophila*-obtained Δγ381 particles and the baculovirus-obtained counterpart allowed us to assess potential capsid stabilizing effects resulting from other molecules such as an endogenous small molecule pocket factor described for the picornaviruses[2] and proposed for the nodaviruses[4].

Relative capsid dynamics were studied by trypsin digestion experiments performed in parallel with all six types of particles (Fig. 1). The rate of proteolysis was determined by the addition of an internal standard (a synthetic version of the γ-peptide consisting of the N-terminal 21 amino acids) to each reaction. Relative ion intensities of the released peptide fragments were used to allow comparisons between reactions. A dramatic difference was seen between v-WT-d and r-WT-b as well as v-D75N-d and r-D75N-b (see Table 1 for abbreviations). The particles containing the viral genomic RNAs were digested much more slowly than the baculovirus-expressed particles containing cellular RNAs. In contrast, both types of Δγ381 particles were digested rapidly and with similar efficiency compared with the particles encapsidating viral RNA, indicating that any stabilizing effects due to pocket factor-like molecules had to be small compared with the effect of authentic RNA.

An alternative method for examining protein accessibility as a function of mobility is chemical modification. The accessibility of protease cleavage sites on the viral capsid is limited by the geometry of the protease active site and is dependent on polypeptide mobility. Higher order protein structure can be detected by the relative reactivities of site specific chemical modification[9,10]. To determine whether the change in virus structural mobility could be detected by a nonenzymatic probe, we used the site specific modification of lysine residues through amino-acetylation. Reactivities of the lysine residues and the N-terminus

Table 1 Six types of FHV particles used to investigate viral capsid dynamics[1]

Name	RNA	Capsid protein	Cell type
v-WT-d	v (viral)	WT (wild type)	d (drosophila)
r-WT-b	r (random)	WT (wild type)	b (baculovirus expressed)
v-D75N-d	v (viral)	D75N	d (drosophila)
r-D75N-b	r (random)	D75N	b (baculovirus expressed)
r-Δγ381-d	r (random)	Δγ381	d (drosophila)
r-Δγ381-b	r (random)	Δγ381	b (baculovirus expressed)

[1]In addition to particles containing wild-type coat protein, two mutants were studied. In one mutant a single amino acid replacement at position 75 (Asp to Asn) created a cleavage-defective particle, D75N. In a second mutant deletion of amino acids 382–407 removed the C-terminal sequence required for recognition of viral RNA during assembly, Δγ381. FHV was either prepared in *Drosophila* cells (d) which produce infectious virions containing viral RNA (v), or the baculovirus expression system (b) which produces morphologically indistinguishable virons that encapsidate random RNA (r) during capsid assembly.

of γ-peptide were lower in v-WT-d than r-WT-b particles (Fig. 2). Although all three primary amines on γ-peptide reacted (two lysines and the N-terminus), the addition of an internal standard revealed that the reaction proceeded more slowly in the v-WT-d particles. This result was in line with the trypsin digestion experiment, which indicated that the v-WT-d particles exhibit less structural mobility than the r-WT-b particles. On the other hand, both versions of the Δγ381 particles reacted equally to amino-acetylation. The internal standard as used in all previous experiments was a synthetic version of the γ-peptide consisting of the N-terminal 21 amino acids. This region forms an amphipathic helix in solution and contains all of the potential acetylation sites. The results from the amino acetylation experiments were consistent with results from the proteolysis experiments and showed that the differences in capsid dynamics are not an artifact of protease accessibility.

Overall, our results show that particles with identical high resolution crystal structures can exhibit significantly different properties in solution. This observation further implies that, in addition to carrying genetic information, the encapsidated RNA also contributes to particle integrity and structural mobility, a dual functionality of viral RNA that could be evolutionarily significant.

Methods

Mass analysis was conducted using a Kratos Kompact and a PerSeptive Biosystems Voyager Elite, both equipped with delayed extraction. All analyses were conducted using 3,5-dimethoxy-4-hydroxycinnamic acid (Aldrich) in a saturated solution of acetonitrile/water (50/50 v/v) 0.25% trifluoroacetic acid. Proteolytic digests were conducted at room temperature on 0.7–1.0 mg ml^{-1} virus for 20 min. Modified Trypsin (Promega), was used at concentrations ranging from 1:10 to 1:40,000 (w:w) enzyme to virus. All digests were in 25 mM Tris-HCl (pH7.7), 1 mM EDTA. Reaction volumes were 10–20 μl and 0.5–1.0 μl was removed for each analysis.

Relative rates of digestion were determined by the ion intensity of released tryptic peptides compared with an internal standard to determine relative concentration. Proteolytic digests were conducted at room temperature on 0.7 mg ml^{-1} virus in 20 μl. Modified Trypsin (Promega), was used at 1:100 (w:w) enzyme to virus. All digests were in 25 mM Tris-HCl (pH 7.7), 1 mM EDTA. After 7 h, 1.0 μl aliquots were removed from each reaction (n = 3) and placed directly on the analysis plate where the reaction was stopped by the addition of matrix which included the internal standard. Mass spectra were the result of 100 (Kompact) or 128 (Voyager Elite) averaged laser pulses. Ion intensity was determined using the recorded counts on the Voyager Elite and by the area under the peak for data from the Kompact. 90% confidence intervals were ±0.22, 0.23, 0.38, 1.0, 1.1 and 1.7 (n = 3).

Acetylation reactions were in 50 mM Tris-HCl (pH 7.6), 1–10% acetic anhydride. The pH was maintained by step-wise addition of 14.75% Ammonium hydroxide to the 1–4 mg ml^{-1} virus solutions. Reactions were complete generally within 30 min and remained stable for 24 h. Before MALDI analysis, samples were diluted to 1 mg ml^{-1} with water.

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