Suppression of a Morphogenic Mutant in Rous Sarcoma Virus Capsid Protein by a Second-Site Mutation: a Cryoelectron Tomography Study^{▽†}

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Retrovirus assembly is driven by polymerization of the Gag polyprotein as nascent virions bud from host cells. Gag is then processed proteolytically, releasing the capsid protein (CA) to assemble *de novo* inside maturing virions. CA has N-terminal and C-terminal domains (NTDs and CTDs, respectively) whose folds are conserved, although their sequences are divergent except in the 20-residue major homology region (MHR) in the CTD. The MHR is thought to play an important role in assembly, and some mutations affecting it, including the F167Y substitution, are lethal. A temperature-sensitive second-site suppressor mutation in the NTD, A38V, restores infectivity. We have used cryoelectron tomography to investigate the morphotypes of this double mutant. Virions produced at the nonpermissive temperature do not assemble capsids, although Gag is processed normally; moreover, they are more variable in size than the wild type and have fewer glycoprotein spikes. At the permissive temperature, virions are similar in size and spike content as in the wild type and capsid assembly is restored, albeit with altered polymorphisms. The mutation F167Y-A38V (referred to as FY/AV in this paper) produces fewer tubular capsids than wild type and more irregular polyhedra, which tend to be larger than in the wild type, containing ~30% more CA subunits. It follows that FY/AV CA assembles more efficiently *in situ* than in the wild type and has a lower critical concentration, reflecting altered nucleation properties. However, its infectivity is lower than that of the wild type, due to a 4-fold-lower budding efficiency. We conclude that the wild-type CA protein sequence represents an evolutionary compromise between competing requirements for optimization of Gag assembly (of the immature virion) and CA assembly (in the maturing virion).

In the first step of retrovirus maturation, the Gag precursor polyprotein is processed into three main components: the matrix (MA), capsid (CA), and nucleocapsid (NC). Of these, ~1,000 to 2,000 copies of CA assemble into a capsid shell, enclosing the dimeric RNA genome and viral replication enzymes, while leaving a sizable population of unassembled CA subunits (5, 24). A properly formed capsid is thought to be essential for infectivity. The CA proteins of different retroviruses are quite uniform in size, ~230 to 240 amino acids, but exhibit little sequence similarity except in the major homology region (MHR). Nevertheless, they share a structure with two

domains (the N-terminal and C-terminal domains [NTDs and CTDs, respectively]) of conserved folds, connected by a short linker (2, 3, 8, 12, 17, 21, 22, 28). The NTDs form hexameric and pentameric rings that associate via homodimeric interactions between CTDs present in neighboring rings. Recently, structural evidence for a third interaction between the NTDs and the CTDs has been presented (10, 16, 31).

The observed conservation of the MHR sequence points to its functional importance; in particular, the MHR is thought to play key roles, both in Gag assembly to produce immature virions and subsequently in the assembly of capsids within maturing virions. Consistent with this, several studies have reported adverse effects of point mutations in the MHR: certain mutations block the assembly of Gag proteins, whereas others have no evident effect on Gag assembly, genome incorporation, or budding but yield virus-like particles that are non-infectious or poorly infectious (1, 7, 13, 26, 30, 35–37). Starting with mutations of the latter kind, a series of second-site suppressors have been isolated that partially restore infectivity. Of these, two mapped in the NTD (A38V and P65Q), three in the dimerization helix in the CTD (F193L, R185W, and I190V), and one in the cleavage site between CA and the downstream peptide (S241L) (4, 7, 13, 25). The rescue of the MHR mutants by suppressing mutations was found not to be allele specific (25). The lack of allele specificity and the temperature sensitivity of some MHR mutations and their suppressors suggest

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that the affected residues are important for achieving a conformation(s) needed for proper assembly.

In a previous study, we used cryoelectron tomography (cryo-ET) to characterize the pleomorphic variability of wild-type Rous sarcoma virions (6, 20). Some 80% of them were found to contain capsids, which could be tubular, irregular polyhedra, or “continuous curvature” capsids, lacking angular vertices. The capsid type was found to correlate with the number of glycoprotein spikes per virion and with the efficiency of assembly, or conversely, the size of the pool of unassembled CA subunits contained within a virion. Based on these observations, we posited that in capsid assembly *in situ*, which is necessarily a nucleated process, different kinds of nucleation complexes initiate assembly of the various kinds of capsids. We also observed that tubular and some continuous curvature capsids had little internal material, suggesting that packaging of the viral ribonucleoprotein (RNP) had failed. Accordingly, and to accommodate the extensive polymorphism that was observed, we posited that a viable core is one with a closed capsid (of any morphology) that has successfully packaged its RNP. Subsequent *in vitro* studies have supported and clarified the perception of CA assembly as a variably nucleated process. Specifically, (i) it has been demonstrated that CA protein can assemble in a nucleation-driven manner into a variety of capsid-related structures (32, 33), and (ii) the mutation F167Y has been found to hamper nucleation of CA assembly *in vitro*, while the A38V suppressor strongly promotes assembly. Thus, CA-A38V assembles exceedingly rapidly, and in the double mutant, the A38V change overcomes the nucleation defect caused by F167Y.

We have now further investigated the relationships among capsid morphology, nucleated assembly, and infectivity by performing a cryo-ET analysis of virions produced by the temperature-sensitive double mutant in which F167Y is complemented with the suppressor A38V; at the permissive temperature, the infectivity of the double mutant is restored to about 70% of wild type (25). Prior observations (32, 33) allowed the prediction that capsid assembly *in vivo* would be impaired in initiation for F167Y and for F167Y-A38V (abbreviated hereafter as FY/AV) at the nonpermissive temperature. Expectations for the double mutant at the permissive temperature were less clear: capsids should be produced, but this process might be altered in some way, as infectivity is lower than in the wild type. Because infectivity as measured could also be affected by Gag-related functions occurring earlier in the replication pathway, i.e., in viral budding or in proteolytic processing, we also compared the rates of virus growth, terminal Gag cleavage, and budding efficiency under these conditions for both the mutants and the wild-type virus.

MATERIALS AND METHODS

Preparation of virions. Wild-type and mutant viruses encoded by the retroviral vector pRS.V8.eGFP, which bears the *gag* gene of the Rous sarcoma virus (RSV) Prague C strain, have been described previously (25). Infectivity was confirmed by transferring the viruses to uninfected DF1 (chicken) cells and monitoring the rate of spread of green fluorescent protein (GFP) expression from the viral genome over 1 to 2 weeks, as described previously (25). For particle production, cells infected with mutant or wild-type RSV, cultured in Dulbecco's modified Eagle medium (DMEM) with 7.5% fetal bovine serum, were seeded at 37°C and 42°C and allowed to adapt for at least 1 week. Cells were then passaged into multiple 100-mm plates. The medium was replaced with low-serum medium

TABLE 1. Parameters of the tomography data set

Tilt series (growth temp [°C], exp. no.)	Tilt range (°)	No. of virions	Resolution ^a (nm)
FY/AV (37, 1)	−55 to 67	36	5.6
FY/AV (37, 2)	−61 to 66	30	5.4
FY/AV (37, 3)	−58 to 62	27	6.4
FY/AV (37, 4)	−51 to 52	28	6.0
FY/AV (42, 1)	−57 to 57	21	5.7
FY/AV (42, 2)	−60 to 61	17	6.0
FY/AV (42, 3)	−58 to 62	11	5.7
FY/AV (42, 4)	−59 to 62	12	5.8
FY/AV (42, 5)	−60 to 60	13	5.7
FY/AV (42, 6)	−51 to 57	5	6.2

^a In-plane resolution, measured according to the NLOO-2D criterion (see Materials and Methods).

(DMEM with 2% of 0.2- μ m-filtered fetal bovine serum) 24 h later. After another 48 h, the medium from each plate was harvested, with care taken to maintain the temperature at 4°C. Harvested medium was clarified by spinning twice at $3,820 \times g$ at 4°C for 5 min to pellet any cell debris. The supernatant was overlaid on a 20% sucrose cushion and spun at $76,755 \times g$ for 70 min at 4°C. The virus pellet was resuspended in a low-salt buffer (10 mM Tris-HCl, 5 mM NaCl; pH 7.6) and stored at 4°C until used for cryo-EM. The amount of CA protein in each virus preparation was estimated by Western blotting.

Pulse-chase experiment. Plates of wild-type and mutant-infected cells maintained at 37°C were pulse-labeled for 15 min with [³⁵S]methionine/cysteine before being shifted to medium containing only unlabeled amino acids. Culture media were harvested at intervals, beginning 15 min after the shift, and CA-related proteins were recovered by immunoprecipitation.

Measurement of critical concentrations. Monomeric CA was expressed in *Escherichia coli* in its native 237-amino-acid form without tags or extensions at either terminus, as described previously (33). Proteins were purified by DEAE and size exclusion chromatography and stored at 10 mg/ml in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 mM EDTA. Assembly was carried out at ambient temperature (~25°C) in 100- μ l samples in a sealed 96-well plate (to help prevent evaporation) and monitored by turbidity (optical density at 450 nm), as described previously (33). After 9 h of incubation, the samples were centrifuged at $128,000 \times g$ for 30 min at 20°C to pellet particulate matter. The concentration of CA remaining in the top 80 μ l of the supernatant was determined based on the A_{280} .

Budding efficiency. Chronically infected DF1 cells (4×10^6 ; wild type [WT], A38V, or FY/AV) were seeded onto 60-mm plates (five plates for each virus). The next day, cells were starved with serum-free medium for 30 min and then labeled with [³⁵S]Met-Cys. After 15 min, the labeling medium was removed and replaced with medium containing cold Met-Cys. Cell lysates from one set of plates were harvested immediately (0-min chase). Cell lysates and medium samples were harvested at the indicated times postchase from appropriate sets of plates. The viral proteins from these samples were immunoprecipitated using polyclonal rabbit antibodies raised against *E. coli*-expressed CA (a kind gift of S. Hughes, NCI—Frederick, and V. Vogt, Cornell University) and subjected to SDS-PAGE in a 15% gel followed by autoradiography. Budding efficiency was measured by calculating the ratio of the CA protein found in media samples (210-min time point) to the level of Gag protein in the cell lysates (0-min time point) and expressed as a percentage of the wild type value.

Cryoelectron tomography. Virions were mixed with a suspension of 10-nm colloidal gold particles (Ted Pella Inc., Redding, CA) to provide fiducial markers. Four-microliter drops were applied to holey carbon films, thinned by blotting, and vitrified in liquid ethane using a Vitrobot (FEI Company, Hillsboro, OR) before transfer into a cryo-holder (model 626; Gatan, Warrendale, PA). Cryo-ET was carried out on a Tecnai-12 electron microscope (FEI, Hillsboro, OR) operating at 120 kV and equipped with a LaB₆ filament, a 2,048- by 2048-pixel charge-coupled-device camera, and a GIF (Gatan) energy filter which was operated in the zero-energy loss mode with a slit width of 20 eV. Low-dose projections (0.5 electrons/Å²) were recorded at 1° steps over a tilt range of typically −60° to 60°, at an effective magnification of $\times 38,000$. Defocus values of ~6 μ m were used, placing the first zero of the contrast transfer function for untilted projections at a spacing of $\sim(4.5 \text{ nm})^{-1}$. Data acquisition used SerialEM (27) to conduct automatic tilting, tracking, focusing, and image recording. The total dose used per tilt series was 60 to 70 electrons/Å².

FIG. 1. Central slices (0.78 nm thick) through cryotomographic reconstructions of RSV viral particles. (A and B) FY/AV mutant virions produced at the nonpermissive temperature of 42°C; (C and D) FY/AV virions produced at the permissive temperature of 37°C. (E) Wild-type virions produced at 37°C; the examples shown here include virions with tubular capsids, one “continuous-curvature” capsid, one polyhedral capsid, and a rare double-layered prismatic capsid.

Image analysis. Projections in a tilt series were mutually aligned with reference to the gold particles, using the IMOD software package (23), which was also used to calculate the tomograms. From them, subvolumes containing individual virions were extracted and denoised by nonlinear anisotropic diffusion (14), as implemented in Bsoft (19). Denoised virions were segmented manually and surface rendered using the Amira software package (Mercury Computer System, Inc.). Capsid surface areas and volumes were also estimated with Amira. The resolution of each tomogram was measured as an average of the values calculated for three particles according to the NLOO-2D criterion, as implemented in ELECTRA, using a threshold of 0.3 (9). The salient parameters of the full tomographic data set are compiled in Table 1.

RESULTS

The strongly temperature-sensitive nature of the FY/AV double mutant provided the opportunity to produce stocks of genetically identical particles in which the effects of mutations are controlled by the temperature at which virions are produced. For this purpose, stable producer lines of chicken DF1 cells infected with the wild-type virus or the double mutant were established as described in Materials

and Methods. The growth properties of the progeny of each culture were confirmed by transferring particles to uninfected DF1 cells and measuring the rate of spread of GFP expression through the culture during exponential virus growth (25). The results verified the robust growth of the wild-type virus at both temperatures and a slight reduction for the mutant at 37°C (~70% of the spreading rate characteristic of the wild type). Furthermore, the first quantitative evaluation of replication of the FY/AV mutant at the nonpermissive temperature was obtained; the rate of spread at 42°C was below the level of reliable measurement (<4% of the rate of wild-type virus at the same temperature in five independent determinations). Stocks of wild-type and mutant virions were produced at the two temperatures for tomographic analysis.

Phenotype of the FY/AV mutant grown at 42°C. The structural alterations in mutant virions produced at the nonpermissive temperature were striking. As represented in a sampling of 79 virions visualized in six tomograms (Fig. 1A and

FIG. 2. (A) Distributions of virion diameters for wild-type RSV virions grown at 37°C and for the FY/AV double mutant grown at 37°C (red) or 42°C (gold). Two FY/AV (42°C) virions with outlier diameters of 209 nm and 265 nm, respectively, were not included. (B) Mean virion diameters and standard deviations.

B), these particles were generally spherical but more variable in size than wild-type virions and they tended to be larger; their average diameter was 134 ± 18 nm (standard deviation; range, 91 to 265 nm), compared to 125 ± 10 nm (range, 110 to 145 nm) for the wild type (Fig. 2). Also, they had markedly fewer glycoprotein spikes, averaging 7 ± 6 (range, 0 to 24) spikes per virion compared to 36 ± 31 (range, 2 to 118) for the wild type (Fig. 3A and B). However, their most conspicuous departure from wild-type morphology was in the almost complete absence of capsids. Whereas ~80% of wild-type virions contain capsids, only 1 of the 79 FY/AV (42°C) virions contained a capsid, which happened to be an open-ended tube detected in an unusually large virion (diameter, 265 nm).

In the absence of the two most evident distinguishing features of RSV virions, internal cores and external spikes, the question arose of whether the particles analyzed were virions or large filled vesicles of cellular origin. The observed thickness of the envelope, which is approximately twice as thick as a lipid bilayer on account of the closely apposed MA layer (see “Some RSV virions have incomplete MA layers” below), attests to the virion-like nature of these particles. Thus, it appears that the

mutant Gag assembles into provirion-like particles and is processed normally, with MA lining the membrane as usual, but CA, although present, does not assemble into capsids.

Phenotype of the FY/AV mutant grown at 37°C. In four tomograms, we visualized a total of 121 mutant virions grown at the permissive temperature. Like wild-type virions, FY/AV virions are pleiomorphic (Fig. 1C and D). However, unlike wild-type virions, a substantial fraction of mutant virions (~30%) depart significantly from sphericity. The most common nonspherical morphology is tear shaped, with an axial ratio of ~1.3 (Fig. 1C, top row, middle panel). The round particles (84, of which 60 contained a core) have a similar size distribution to wild-type virions (average diameter, 120 ± 7.7 nm; range, 98 to 161 nm) (Fig. 2). One of these is rendered in Fig. 3C and D and can be compared with a wild-type virion, similarly rendered, in Fig. 2b of reference 6. It is unlikely that the nonspherical morphologies represent a purification artifact, as they are much rarer (<5%) in wild-type isolates prepared in the same way. An example of a nonspherical mutant virion is rendered in Fig. S1 of the supplemental material.

The majority of glycoprotein spikes, which we take to be Env trimers, are similar to those present on the surface of wild-type virions (6). We also observed a few spikes with different shapes that are presumably molecules of cellular provenance (34) (Fig. 1C, red arrow). Upon mapping the spike locations on the surfaces of 94 virions (84 spherical and 10 nonspherical virions), we found them to be distributed in dispersed clusters but with no local or global order (Fig. 3C and D). The number of spikes per virion averaged 43 ± 20 (range, 6 to 107) (Fig. 3A and B). There was no evident correlation of spike number with virion size, except that some of the smaller virions had more spikes than other virions.

As for cores, the suppressing AV mutation restored the fraction of capsid-containing virions to wild-type levels. A total of 76% of these virions (92 of 121) had a single-layered capsid. The remainder either lacked capsids ($n = 13$) or had incomplete capsids ($n = 14$) or double-layered capsids ($n = 2$). The mutant capsids exhibited, like the wild type, a high degree of polymorphism but with a different distribution of structures. In particular, there were more irregular polyhedra than the wild type (~71% versus 22%), fewer “continuous curvature” capsids (~28% versus 52%), and almost no tubular capsids (~1% versus 28%). The respective distributions of capsid types are compared in Fig. 4.

In addition to their increased incidence, the polyhedral capsids tended to be larger than those of wild-type virions. The latter property was apparent from visual inspection of central sections (cf. Fig. 1C and D with E). This observation was confirmed quantitatively by measuring their volumes and surface areas (Fig. 5A and B). FY/AV capsids have, on average, ~30% more CA than wild-type capsids.

To confirm that the observed effects could be attributed to the mutations involved rather than elevated growth temperature, we also recorded tomograms of wild-type virions grown at 42°C and compared them with virions grown at 37°C. These data (not shown) indicated that the incidence of capsid-containing virions and their polymorphism are much the same at both temperatures.

FY/AV CA assembles more efficiently *in situ* than wild-type CA. In addition to assembled capsids, retrovirions contain pop-

FIG. 3. (A) Distributions of numbers of glycoprotein spikes on spherical RSV virions, as determined for wild-type RSV grown at 37°C and for the FY/AV double mutant grown at 37°C (red) or 42°C (gold). (B) The corresponding mean numbers of spikes $\pm$ standard deviations (44 ± 21 and 7 ± 6 , respectively, for FY/AV and 36 $\pm$ 31 for wild-type virions). (C) Surface rendering of the outer surface of a spherical FY/AV virion grown at 37°C. Protruding glycoprotein spikes (92 in all) are grouped in local clusters, separated by bare patches of membrane. (D) Cutaway view exposing the virion's interior: it reveals the capsid (red), encapsidated material which presumably includes the genomic RNA/NC complexes (orange), and material between the capsid and envelope (gray), which should include some unassembled CA subunits. Scale bar, 50 nm.

ulations of unassembled CA subunits (5, 24). The tomograms allowed us to estimate CA assembly efficiency for individual virions. Efficiency is given by the number of CA subunits in the capsid, N_{CA} , considered a fraction of the total number of CA subunits available, which was taken to be the same as the number of Gag subunits, N_{Gag} , incorporated during assembly. As described previously (6), we determined N_{CA} from the surface area of the capsid and N_{Gag} from the diameter of the mature virion. In Fig. 6A, each virion is represented as a point in a two-dimensional plot with coordinates (N_{CA} and N_{Gag}). If assembly of a given capsid were to be 100% efficient, its point

would lie on the diagonal ($N_{CA} = N_{Gag}$). As efficiency falls off for virions of a given size (i.e., a given value of N_{Gag}), the data points move toward the y axis, parallel to the x axis. The numbers of assembled and unassembled CA subunits for each virion may be read off this plot, as illustrated in Fig. 6A.

According to this analysis, FY/AV virions vary substantially in their assembly efficiency; some virions assemble almost all of their CA subunits into their capsid and some assemble as few as ~30%. A similar variability is exhibited by wild-type virions; however, it is apparent that the FY/AV mutant CA subunit assembles more readily than the wild type, i.e., its cloud of data

FIG. 4. Pie charts showing the percentages of RSV virions with various classes of cores for both wild-type virions ($n = 95$) and double mutant FY/AV virions produced at 37°C ($n = 121$).

points in Fig. 6A is closer to the diagonal. The average assembly efficiency for spherical virions was estimated to be 72%, compared with 52% for the wild type (6).

Some RSV virions have incomplete MA layers. The foregoing analysis assumes that virions assemble complete Gag shells. If they were to be incomplete, the estimated numbers for N_{Gag} (and for the assembly efficiencies) would need to be changed accordingly. In this context, tomographic studies of immature HIV virions have documented sizeable gaps in their Gag shells (11, 38). We have not yet succeeded in visualizing immature RSV virions, but observations on mature RSV virions suggest that they are subject to a similar effect. On some FY/AV virions, we noticed patches of envelope that were thinner than the norm (Fig. 6B and C). On revisiting our tomograms of wild-type RSV virions (6), we found that some of them had similarly thin patches. Unlike influenza virus, whose matrix protein layer is well resolved from the lipid bilayer in cryotomograms (18), RSV MA is tightly apposed to its bilayer and is not resolved. (In comparison, for HIV, the bilayer and the MA layer are at least partly resolved [38; P. Keller, personal

communication].) We surmise that the thinner patches of envelope represent MA-free regions inherited from immature virions with incomplete Gag layers.

The conditions for distinguishing thick from thin areas of envelope are most favorable in central sections of reconstructed virions. (In the “near” and “far” parts of the tomograms, the definition of the envelope is compromised by “missing wedge” effects [15].) A validation of our ability to reproducibly distinguish between thick and thin sectors of viral envelope is given in Fig. S2 of the supplemental material. If the region of MA-free membrane is approximated as a circular cap (11, 38), then the issue of whether and how fully it will be sampled in a central section depends on how the cap is oriented relative to the sectioning plane. Nevertheless, we may make an overall estimate of the size of the putative MA-free caps by measuring the arc length of thin (MA-free) envelope for each virion and averaging these data. On this basis, we obtained $50^{\circ} \pm 19^{\circ}$ for wild type and $39^{\circ} \pm 16^{\circ}$ for FY/AV virions, while expecting there to be some variability from particle to particle (see below). If

FIG. 5. (A) Distributions of surface areas of intraviral RSV capsids for the wild-type and the FY/AV mutant at 37°C . The mutant capsids had, on average, significantly larger surface areas (the red histogram is displaced relative to the green histogram). (B) The distributions shown convey two measurements for each virion: internal volume of the capsid (x coordinate) and that volume expressed as a fraction of the virion internal volume (y coordinate).

FIG. 6. (A) Copy numbers of CA subunits assembled into capsids (N_{CA}) and total numbers of CA subunits (N_{Gag}), calculated from the tomograms, are plotted on a per virion basis. Data are shown for 74 wild-type capsid-containing RSV virions and for 60 FY/AV RSV virions grown at 37°C. The bold diagonal line represents situations in which all CA subunits assemble, i.e., $N_{CA} = N_{Gag}$. The region to the right of this line, $N_{CA} > N_{Gag}$, represents an unphysical situation, as a capsid cannot assemble more CA subunits than are available within the maturing virion. The region to the left, $N_{CA} < N_{Gag}$, is populated by virions that also contain pools of unassembled CA subunits. The contents of assembled and unassembled CA subunits for one virion (top left) are marked as an example. In the first instance, we assumed that all Gag shells were complete. However, it is likely that some or all Gag shells were incomplete when the immature virion budded from the host cell (see text). The dashed line gives the revised diagonal if the Gag shells were only 90% complete, which we estimate to be the average situation, and the dotted line corresponds to 20% incompleteness. (B and C) Two examples of central sections of RSV virions that have visibly thinner regions in their envelopes (marked with arcs), compared to the norm. We interpret the thicker (normal) envelope regions as the lipid bilayer lined with a layer of matrix protein and the thinner regions as patches of MA-free bilayer (38).

these numbers from arc lengths when measured in two dimensions from central sections are converted to percentages of the spherical surface areas occupied by circular caps, they may be calculated to be $8\% \pm 5\%$ and $5\% \pm 3\%$, respectively.

The effect of incomplete Gag shells on our calculations of assembly efficiency is to shift the diagonal, as illustrated in Fig. 6A for the cases of 10% incompleteness (dashed line) and 20% (dotted line). These shifts have the effect of moving some data points across the diagonal to the region where $N_{CA} > N_{Gag}$, which is physically impossible unless assembling virions were to incorporate additional Gag molecules inside a complete shell, an eventuality that we consider unlikely. We infer, therefore, that the putative unfilled patch of the Gag shell is variable in size and is very small in some RSV virions, as the foregoing analysis implies. Taking its average size over the population as a whole as 10%, the estimated contents of unassembled CA subunits per virion are correspondingly reduced, and the average assembly efficiencies increase to 90% (FY/AV) and 58% (wild type).

Effects of temperature and mutation on Gag processing and budding efficiency. Nucleated assembly of capsids *in situ* necessarily depends on the rate at which building blocks are generated. Accordingly, we performed a pulse-chase experiment to compare wild-type and mutant virus at 37°C for their rates of cleavage in the final Gag processing step, in which CA-SP (which carries at its C terminus the spacer peptide that separates CA and NC) is cleaved to the CA (237 amino acids) and

CA-S (240 amino acids) forms found in mature virions (see Materials and Methods). The form of CA detected shortly after initiating the chase is primarily the larger CA-SP, which is gradually cleaved to CA and CA-S (Fig. 7A and B). A38V caused no obvious difference in the kinetics of this reaction, either alone or when combined with F167Y. In each case, CA-SP is essentially fully processed to CA and CA-S by 3.5 h (data not shown). Likewise, comparison of the intensity of the CA bands in cell lysates with the Gag band indicated no obvious difference in the rate at which CA-SP is released from Gag; nor was any accumulation of abnormal cleavage products seen (Fig. 7A). We conclude that there is no significant difference between the FY/AV mutant and wild-type virus in the rates at which they process Gag. As the need for frequent manipulations of the infected cells during this experiment made it difficult to maintain precise control of the temperature, we did not attempt to compare the processing rates at permissive and nonpermissive temperatures.

Infectivity might also be affected by changes in budding efficiency, which is largely a property of Gag and may therefore be affected by mutations in CA. Budding efficiency could be calculated from data obtained in the previous experiment. Gels were subjected to phosphorimaging analysis, and the relative amounts of radioactivity in the triplet of CA species and in Gag were determined. For each virus, the total amount of CA species detected in medium at 210 min was corrected for the amount of Gag present in lysates at 0 min (the end of the 15-min labeling period). This ratio was then set to 100% for the

FIG. 7. Pulse-chase experiments monitoring Gag cleavage in wild-type and FY/AV RSV particles. Infected cells were pulsed with [³⁵S]Met-Cys for 15 min and then shifted to unlabeled medium. Cell lysates and culture media were harvested at intervals thereafter, and CA-related proteins were recovered by immunoprecipitation with anti-CA serum. (A) Gag and CA profiles displayed in a run in which the CA triplet (CA-S, CA, and CA + SP) migrated ~40% of the gel length (the bottom part of the gel has been truncated). Times indicate the lengths of the chase period. The shift from the CA-SP band to the CA-S/CA doublet is apparent, taking place mainly between 15 and 60 min. The corresponding profiles for A38V particles (not shown) were indistinguishable from those of WT and FY/AV. (B) In a separate experiment, the triplet bands were better resolved, with CA-SP running below the doublet of the other two proteins on this extra-long run on a 13-cm 15% acrylamide gel in which the triplet migrated ~60% down the gel.

wild-type virus. The A38V substitution caused a modest decrease in budding efficiency to 75% (although with considerable variability, ±43%, among replicates), consistent with its mild effect on virus replication (see above). The F167Y substitution alone enhances virus budding (150% of wild-type rate [7]). However, when A38V is combined with the F167Y allele, a surprisingly strong net defect in budding efficiency is registered (down to 28% ± 8%).

Assuming that budding efficiency provides an indirect measure of Gag function, we conclude that, in the context of the double mutant, A38V has a negative effect (presumably acting upon Gag structure and packing) that compromises the efficiency of immature particle formation/release. The inferred effect on Gag appears consistent with the altered particle size distribution (i.e., larger particles) at 42°C and the higher incidence of nonspherical particles.

F167Y/A38V has a lower critical concentration for *in vitro* assembly than wild-type CA. Previously, we observed that the surface areas, and hence, CA copy numbers, of tubular capsids in wild-type RSV virions were lower than those of polyhedral capsids (6). Accordingly, other factors (virion size and completeness of Gag shell) being approximately equal, the pools of unassembled CA subunits should be higher inside virions with tubular capsids. The size of this pool reflects the critical concentration for assembly, there being, by inference, different nucleation mechanisms employed in the assembly of tubular and polyhedral capsids. In the present study, we found that polyhedral FY/AV capsids tended to be substantially larger than in the wild type. Applying the same reasoning as above, it follows that the pools of unassembled subunits and the critical concentration for assembly should be correspondingly lower. We tested this prediction by comparing the critical concentrations for *in vitro* assembly of wild-type CA and the double mutant.

The proteins were expressed and purified and assembly was induced by adding sodium phosphate to give a final concentration of 500 mM at 150 μM protein (33). After 9 h of incubation (to allow assembly to reach the maximum possible), the reaction mixtures were centrifuged to pellet particulate matter. Under these conditions, a variety of capsid-related

structures (tubes, spheres, polyhedral structures, and multilayers) are produced with both wild-type CA and the double mutant protein (33). The concentration of CA remaining in the supernatant, representing the critical concentration, was determined by measuring the absorbance at 280 nm. The results (Fig. 8) are consistent with our other data. The critical concentration for FY/AV CA is almost 3-fold lower than for wild-type CA. For the single mutant F167Y, it is approximately 6-fold higher than the wild type, consistent with the failure of this mutant to produce capsids *in situ*. For the single mutant A38V, it is 4-fold lower than the wild type, and this phenotype overrules the deficit in F167Y when the two mutations are combined.

DISCUSSION

Based on prior *in vitro* assembly studies, we expected that capsid assembly *in situ* would be impaired at the stage of nucleation for FY/AV virions produced at 42°C. The tomograms show that capsid assembly is, in fact, essentially obviated under these conditions. In addition to lacking capsids, these virions tend to be larger than the wild type, indicating that Gag

FIG. 8. Critical concentrations for *in vitro* assembly in the presence of 0.5 M PO₄ for wild-type RSV CA protein and three mutants and for the wild-type protein in the absence of PO₄.

assembly is also affected. Moreover, these virions have few spikes, a property that may also reflect a conformationally altered Gag protein (29). Previously, we observed that wild-type virions whose capsids are open-ended tubes have few spikes (6); on this basis, we hypothesized that Env endodomains contribute, directly or indirectly, to complexes that nucleate assembly of polyhedral capsids. In this context, the compromised FY/AV particles produced at the higher temperature may be seen to be doubly disadvantaged, suffering from a dearth of spikes as well as a nucleation-reluctant CA subunit.

Mutually offsetting effects of the suppressor mutation at the permissive temperature. The second-site AV mutation restores RSV infectivity largely but not completely (25). Based on morphology, these virions exhibit the same size distribution as wild-type virions and they have as many spikes, indicating that these Gag-related properties are restored to wild-type levels. However, the polymorphic range of these capsids differs from the wild type. In terms of the working hypothesis (see the introduction) that a viable capsid is any closed capsid that has packaged RNP, FY/AV (37°C) appears superior to the wild type, having ~65% closed and filled capsids compared with ~30% for the wild type. These numbers suggest that FY/AV (37°C) should be more infective than the wild type. However, when the budding deficit of the double mutant (~28% of wild type) is factored in, the observed infectivity of FY/AV (~70% of the wild type at 37°C) is in reasonable agreement with the phenotypic characterization recorded here.

The behavior of the FY/AV double mutant underscores the need for balance in meeting the respective requirements for assembly of the immature particle (Gag assembly) and for the later event of capsid formation (CA assembly). Previous studies demonstrated that the F167Y allele alone increases budding efficiency above that of the wild type but severely reduces infectivity by impairing capsid assembly (7, 33). Combining it with the A38V allele restores capsid assembly and infectivity, even though the double mutant is adversely affected in virus budding (i.e., Gag assembly), an effect not seen with either allele alone. Thus, the primary sequence of wild-type CA can be viewed as representing an evolutionary compromise that balances these dual requirements. A further implication is that the roles played by these residues in immature versus mature assembly are different.

CA assembly *in situ* and *in vitro*. It is difficult to directly compare CA assembly *in situ* with assembly *in vitro*, as the concentrations involved are higher, molecular crowding effects are operative, and other potentially reactive molecules are present. Nevertheless, it is noteworthy that electron tomography indicated that the FY/AV mutant leaves a much smaller pool of unassembled CA subunits than does the wild-type virus (2- to 4-fold less, on average, depending on how the completeness of the Gag shell is considered). The size of this pool may be considered as an “effective” critical concentration for assembly. A similar ratio was determined for the critical concentrations of the mutant and wild-type CA *in vitro* assembly experiments (see above).

Locations of the mutated residues. The primary MHR mutation F167Y affects an internal hydrophobic residue in the CTD, whereas the suppressing mutation A38V affects a residue in helix $\alpha 2$ of the NTD. From cryo-EM reconstructions of icosahedral CA capsids assembled *in vitro* (10), we were able to

FIG. 9. Map of mutated residues on RSV CA in multimeric assemblies. (A and B) Top views of a hexamer (A) and a pentamer (B). The structures were obtained from docking crystal structures into cryo-EM density maps of icosahedral particles with triangulation numbers $T = 3$ and $T = 1$, respectively (10). Models of the NTD and CTD are green and blue, respectively. The F167 residues are marked with gold balls, and the A38 residues are marked with red balls. Dashed lines connect the C terminus of the NTD construct modeled to the N terminus of the CTD of the same subunit. (C) Close-up view of the NTD-CTD interface, as in the pentameric ring. Helices in the proximity of this interface are labeled.

construct models of the hexamer and pentamer that show the dispositions of the affected residues in these oligomers (Fig. 9). However, the mechanisms whereby mutations at these sites impose their phenotypes are not obvious. Nevertheless, as residue 167 lies quite close to the NTD-CTD interface, it may be that this mutation affects the propensity of CA to establish NTD-CTD contacts, possibly by altering the conformation of the NTD docking site, which is defined by the first, second, and fourth helices of the CTD. Conversely, A38V may allow a reorganization of the NTD that reestablishes the ability to form NTD-CTD contacts. Alternatively, it may compensate for the F167Y mutation by strengthening the NTD-NTD interface. Such an effect is hinted at in the crystal structure of the HIV CA hexamer (31), where the CTD is seen to interact not only with helix 4 but also with helices 3 and 7. The phenotype of the double mutant (more irregular polyhedra and fewer continuous curvature capsids) could be explained if the inferred modifications at the NTD-CTD interface were not able to restore the original flexibility but instead limit the dihedral angles to a more restricted range of values.

To take this line of argument one step further, it may be that CA assembly starts with the formation of a nucleating oligomer containing an NTD-CTD interface (32). Also consistent with this hypothesis is the fact that several other suppressors affect residues positioned on the second CTD helix and presumably act by restoring a functional CTD conformation; in particular, the F167Y mutation is suppressed by two different point mutations on helix 2 of the CTD, I190V and R185W (4, 25).

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