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Processing of the L1 52/55k protein by the adenovirus protease: a new substrate and new insights into virion maturation

Running title: Cleavage of L1 52/55k by the adenovirus protease

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Abstract

Late in adenovirus assembly, the viral protease (AVP) becomes activated and cleaves multiple copies of three capsid and three core proteins. Proteolytic maturation is an absolute requirement to render the viral particle infectious. Here we show that the L1 52/55k protein, which is present in empty capsids but not in mature virions and is required for genome packaging, is the seventh substrate for AVP. A new estimate on its copy number indicates there are about 50 molecules of the L1 52/55k protein in the immature virus particle. Using a quasi *in vivo* situation, *i.e.* the addition of recombinant AVP to mildly disrupted immature virus particles, we show that cleavage of L1 52/55k is DNA-dependent, as is the cleavage of the other viral precursor proteins, and occurs at multiple sites, many not conforming to AVP consensus cleavage sites. Proteolytic processing of L1 52/55k disrupts its interactions with other capsid and core proteins, providing a mechanism for its removal during viral maturation. Our results support a model in which the role of L1 52/55k protein during assembly consists in tethering the viral core to the icosahedral shell, and in which maturation proceeds simultaneously with packaging, before the viral particle is sealed.

Introduction

Adenovirus morphogenesis ends with a maturation step comprising proteolytic cleavage of several capsid and core precursor proteins. Without these cleavages, the immature particle lacks infectivity because of its inability to uncoat (1-3). Maturation primes the viral particle for stepwise uncoating by facilitating penton release and by loosening the condensed genome and its attachment to the icosahedral shell (4, 5). Proteolytic processing is carried out by the adenovirus protease (AVP, or L3 23K protein) (6). In human adenovirus type 2 (HAdV-2), AVP recognizes (M/I/L)XGX-G and (M/I/L)XGG-X sequence motifs (7, 8). These sequences are present in the precursor proteins pIIIa, pVI and pVIII in the icosahedral shell, as well as in the DNA binding polypeptides pVII, pre-μ, and the terminal protein. These six precursor proteins have been shown to be substrates for AVP. Another potential substrate, because it contains an AVP consensus sequence motif, is polypeptide L1 52/55k.

The L1 52/55k protein in HAdV-2 is 415 residues in length, with an AVP consensus cleavage site at the 351-352 position (LAGT-G). Although the molecular mass of L1 52/55k calculated from its sequence is 47 kDa, the protein was named by its electrophoretic mobility; it moved as a doublet due to two different phosphorylation states (9). L1 52/55k is part of the genome packaging machinery, together with polypeptides IIIa, IVa2, L4 33k, and L4 22k (10-15). An L1 52/55k deleted construct produces only empty capsids (10), and a thermosensitive mutation in the L1 52/55k C-terminal region (*ts369*; 333-EL-336 to 333-GP-336) causes partial packaging (16). L1 52/55k binds to the viral packaging sequence *in vivo* and to the putative packaging ATPase IVa2 *in vitro* (17-19). L1 52/55k contributes to the specificity of packaging, possibly *via* an interaction with capsid protein IIIa (12, 20). Furthermore, L1 52/55k has been reported to bind non-specifically to DNA, and to interact with pVII and its mature form VII in infected cells (21). The interaction with DNA may not be direct, because L1 52/55k does not bind to the DNA packaging sequence *in vitro* (17-19).

L1 52/55k has been considered a putative scaffolding protein, because it is present in empty particles in its full length form, but is absent from the mature virion (9). It is not a *bona fide* scaffolding protein, however, since capsids of apparently the same size and composition as empty wildtype particles assemble in its absence (10). Fully packaged, immature particles produced by the HAdV-2 thermosensitive mutant *ts1* at non-

permissive conditions contain precursor versions of all AVP targets because the virus particles do not contain AVP (3). They have been reported to also contain 2 copies of full length L1 52/55k, compared to ~4 copies in partially packaged capsids and 50 in empty capsids (9). Bands corresponding to molecular weights of 40 and 34 kDa are also recognized by antibodies generated against L1 52/55k in empty or partially packaged capsids (9, 10, 22)[1]. The 34 kDa and some smaller protein species are also revealed in overloaded Western blots of mature particles. The 40 kDa band would correspond to the expected product of proteolysis by AVP at the consensus cleavage site. Both the full length and 40 kDa bands disappear and are replaced by higher molecular weight species in electrophoresis performed in the absence of β-mercaptoethanol, suggesting the formation of disulfide-linked homodimers mediated by the only Cys residue (Cys24) in L1 52/55k (9, 22). The 34 kDa band has been proposed to originate by an additional cleavage at the N-terminus, because it does not react with antibodies against either residues 9-22 or 402-415 in L1 52/55k, and its electrophoretic mobility is not sensitive to non-reducing conditions, consistent with the absence of Cys24 (9, 22). However, the actual processing of L1 52/55k by AVP has not been experimentally observed, nor is the origin of the smaller bands recognized by anti L1 52/55k antibodies known. Furthermore, it is not known what possible role the cleavage by AVP might have in the ejection of the L1 52/55k protein and its fragments from the particle upon packaging of the genome.

AVP has a unique mode of action. It is synthesized as an inactive enzyme (23). It uses two cofactors for maximal enzyme activity: the 11-amino acid peptide pVIc, derived from the C-terminus of the polypeptide VI precursor pVI, and the viral DNA. AVP is activated when pVI slides on DNA into AVP bound to the same DNA (24). AVP, partially activated by being bound to DNA, cleaves pVI at its N-terminus and then at its C-terminus. Cleavage at the C-terminus releases pVIc, which then binds to the AVP that cut it out. The AVP-pVIc complex then slides along DNA, processing the virus precursor proteins also bound to the DNA (25). These experiments were done with fully disrupted *ts1* particles after heating at 60ºC (26). When immature *ts1* particles are incubated at 47ºC for 10 min, they release pentons and peripentonal hexons, but retain

[1] Notice that the bands described as 40 and 34 kDa in (Hasson *et al.*, 1992) are instead described as 47 and 40 kDa in (Sutjipto *et al.*, 2005). Here we use the nomenclature in (Hasson *et al.*, 1992) to avoid confusion with the calculated molecular mass of the full length L1 52/55k protein (47 kDa).

105 the spherical capsid arrangement (27). The viral genome remains inside the "whiffle
106 ball" particle in a highly condensed form attached to the shell (4). Since these mildly
107 disrupted particles have a structural organization close to that of the intact immature
108 capsid, while presenting openings that allow recombinant AVP access to its substrates
109 bound to the viral DNA, we now use them as an experimental system to follow the
110 action of AVP in detail in a more "quasi-*in vivo*" situation. These assays reveal that L1
111 52/55k is a previously unrecognized substrate for AVP, and provide new insights into
112 its role during viral maturation.

113

Materials and Methods

Materials

The gene for AVP was cloned and expressed in *Escherichia coli,* and the protein was purified as described (26). Immature virus was obtained by propagating the HAdV-2 *ts1* mutant in HeLa cells at 39.5º as described (27). Particles were purified by equilibrium centrifugation in CsCl gradients, desalted on a 10DC column (Bio-Rad) and stored in 20 mM Hepes pH 7.8, 150 mM NaCl plus 10% glycerol at -70ºC at a final concentration of $1x10^{13}$ viral particles per ml (vp/ml). An E1-deleted HAdV-5 vector (Ad5GL) was used as a mature, structurally wild-type virus control (28). The following antibodies were used for immunoassays: rat polyclonal anti-pVII (29); mouse anti-V serum (30); rabbit anti-VIII N-terminal fragment (provided by U. F. Greber); rabbit anti-pVI (31); rabbit anti-L1 55/52k (17); and rabbit anti-HAdV-5 fiber knob (32).

Analysis of AVP function in disrupted *ts1* virus

Immature *ts1* virus at a concentration of $1.6x10^{12}$ vp/ml were mildly disrupted by heating at 47°C for 10 minutes, then incubated with 0.25 µM AVP at 37ºC for the indicated times. Reactions were carried out in 10 mM Tris-HCl (pH 7.4), 20 mM NaCl, 10 mM EDTA and stopped by adding electrophoresis loading buffer. This is a suboptimal condition for enzyme activity (better nearer pH 8) (26) and for one-dimensional diffusion on DNA (better nearer pH 6) (25), but it allowed both sliding and enzyme activity to occur at a rate that best revealed details of the maturation process.

To assess the role of DNA in L1 52/55k processing by AVP, viral DNA was removed from the samples as follows. Viruses were either mildly or completely disrupted by heating at 47ºC or 60ºC respectively for 10 min, and incubated overnight at 37ºC with 50 µg/ml DNase I (SIGMA D5025) in 10 mM Tris-HCl (pH 8.2), 5 mM $MgCl_2$. The DNase was inactivated by adding 10 mM EDTA. After 30 min, 0.25 µM AVP was added, and the samples were incubated at 37ºC for 24 hours. In the indicated cases, purified *ts1* DNA was added after DNase inactivation at a final concentration of 50

ng/ml. For DNA isolation, $25x10^{10}$ *ts1* viral particles were treated with proteinase K at a final concentration of 400 µg/ml, and the DNA was extracted by phenol/chloroform precipitation.

Protein electrophoresis and Western blot analysis

To separate viral proteins, samples were boiled in Laemmli loading buffer and subjected to electrophoresis under denaturing conditions (SDS-PAGE) in either 15% or 4–20% (BioRad mini-Protean TGX) acrylamide gels. For Western blot analysis, proteins resolved *via* SDS-PAGE were transferred to PVDF membranes and probed with the required antibodies. Bound antibodies were detected with the corresponding secondary antibody conjugated to alkaline phosphatase (Sigma), and the membranes were developed using an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA) as recommended by the manufacturer.

Protein identification by mass spectrometry

Protein bands observed in SDS-PAGE were carefully excised from Coomassie-stained 4-20% acrylamide gradient gels and subjected to in-gel trypsin digestion according to (33). The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 µg/ml of trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in an ice bath. After 30 min, the supernatant was removed, and 20 µl of 50 mM NH_4HCO_3 were added to the gel pieces. Digestion was allowed to proceed at 37 °C overnight. The reaction was stopped by adding a mixture of 50% ACN and 0.5% TFA. The extracted peptides were dried by speed-vacuum centrifugation and resuspended in 4 µl of MALDI solution (30% ACN + 15% isopropanol + 0.5 % TFA). 20% of each peptide mixture was deposited onto a 384-well OptiTOF™ Plate (Applied Biosystems, Framingham, MA, USA) and allowed to dry at room temperature. A 0.8 µl aliquot of matrix solution (3 mg/mL α-cyano-4-hydroxycinnamic acid in MALDI solution) was then added and allowed to dry at room temperature.

Samples were automatically analyzed in an ABI 4800 MALDI TOF/TOF mass spectrometer (ABSciex, Framingham, MA, USA) working in positive ion reflector

mode (ion acceleration voltage was 25 kV for MS acquisition and 2 kV for MS/MS). Peptide mass fingerprinting and MSMS fragment ion spectra were smoothed and corrected to zero baseline using routines embedded in the ABI 4000 Series Explorer Software v3.6. Internal and external calibration allowed to reach a typical mass measurement accuracy of <25 ppm. To submit the combined PMF and MS/MS data to MASCOT software v.2.1 (Matrix Science, London, UK), GPS Explorer v4.9 was used.

Negative staining electron microscopy

Virus samples were heated at 47ºC for 10 min, and incubated with either AVP 0.25 µM in 10 mM Tris-HCl (pH 7.4), 20 mM NaCl, 10mM EDTA or only buffer for the indicated times. Proteolysis was stopped by adding NaCl to a final concentration of 150 mM. Samples were adsorbed for 5 min onto glow-discharged collodion/carbon-coated copper EM grids. The grids were transferred to a 2% uranyl acetate drop for negative staining, dried and examined in a JEOL JEM 1011 transmission electron microscope.

Quantification of L1 52/55k copy number in *ts1* particles

For semi-quantitative determination of the amount of L1 52/55k protein in different viral populations, identical quantities of purified wildtype or *ts1* capsids (estimated by absorbance at 260 nm) were electrophoresed and silver stained, or probed with anti-V or anti-L1 52/55k antibodies for Western blot as described above. Band intensities in the silver stained gel images were measured with ImageJ (34). The area below each peak in the intensity plot was measured and corrected for the background intensity measured in a nearby area. Since in denaturing gel electrophoresis the full length L1 52/55k protein runs at the same position as polypeptide V, to estimate the copy number of L1 52/55k in full *ts1* viral capsids, we compared the intensity ratio between bands corresponding to polypeptides pVII (VII in wildtype) and V, or IX and V in *ts1* and wildtype virions. Given that Western blot assays indicated that L1 52/55k was not present in wt (**Supplementary Fig. 1**), an estimation of the L1 52/55k content was derived from the excess intensity in the *ts1* polypeptide V band.

Southern blot

Detection of viral DNA by Southern blotting was performed by standard methods (35). Briefly, DNA was extracted from purified *ts1* virus by proteinase K-SDS digestion followed by phenol-chloroform extraction. Purified DNA was electrophoresed on 1% agarose gels, transferred to Hybond nylon filters, and probed with DraIII-digested *ts1* DNA labeled with alkaline phosphatase in conjunction with chemiluminescent detection with CDP-star (Amersham, RPN3680).

Far Western blot

Different fragments of L1 52/55k were obtained by AVP cleavage in disrupted *ts1* capsids, followed by SDS-PAGE and extraction from gel as follows: immature *ts1* virus particles at a concentration of $1.6x10^{12}$ vp/ml were mildly disrupted by heating at 47°C for 10 minutes, then incubated with 0.25 µM of AVP at 37ºC in 10 mM Tris-HCl (pH 8.2), 1 mM EDTA for 24 hours. The sample was electrophoresed under denaturing conditions (SDS-PAGE) in 4–20% acrylamide gradient gels. Unstained bands corresponding to L1 52/55k proteolysis products were excised using as a reference the stained first and last lanes of the gel. The gel pieces were washed 3 times and maintained in elution buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA; pH 7.5) in a rotary shaker at 30°C overnight, then centrifuged at 10,000*g* for 10 minutes.

Proteins present in the supernatant were labeled using two methods. In the first method, proteins were incubated overnight with rabbit anti-L1-52/55k in a rotary shaker at 4°C and cross-linked by adding 0.5% formaldehyde. Protein-antibody complexes were separated from free antibody by Sephacryl S-200HR gel filtration chromatography. In the second method, proteins were incubated with sulfo-NHS-biotin overnight, and the reaction was stopped by adding 10 mM Tris, pH 7.4. The excess biotin reagent was removed using a desalting column.

Samples of mildly disrupted *ts1* virus, digested or not digested with AVP, were electrophoresed and transferred to a nitrocellulose membrane as described for Western blots. For renaturation, the membranes were washed and incubated overnight in refolding buffer TBS-T (100 mM Tris HCl pH 7.6, 150 mM NaCl and 0.01% Tween-20), with 10% glycerol and 5 mM 2-mercaptoethanol. Membranes were then blocked

with 0.05% Tween20 in PBS for 2 hours, BSA 1% (w/v) in PBS for another 2 hours, and incubated with the labeled proteins overnight, at 4ºC with agitation. Finally, the membranes were incubated with either secondary antibody or streptavidin-HRP for 1 hour, and the bound probes were detected using an enhanced chemiluminiscence method.

L1 52/55k release assay

Immature *ts1* particles at a concentration of $1.6x10^{12}$ vp/ml were mildly disrupted by heating at 47°C for 10 minutes, then incubated overnight at 37ºC with either 0.25 µM AVP in 10 mM Tris-HCl (pH 8.2), 10 mM NaCl, 10 mM EDTA or only buffer . The samples were centrifuged at 20,200 g for 60 minutes at 4ºC. The supernatant was collected and concentrated using a speed-vacuum concentrator, and the sediment was dissolved in 10 mM Tris-HCl (pH 8.2), 10 mM NaCl, 10 mM EDTA, before being analyzed by SDS-PAGE, Western blot, or native electrophoresis in agarose gels.

Results

Processing of proteins in mildly disrupted *ts1* particles by AVP

Immature *ts1* particles (containing all protein precursors) were partially disrupted by heating at 47°C for 10 minutes, and incubated with recombinant AVP as described in Materials and Methods. In the reactions, there were approximately 94-100 AVP molecules per viral particle, a ratio similar to that encountered *in vivo* (50 AVP/particle) (36). Processing of the viral proteins was followed by denaturing electrophoresis and by Western blot analysis to identify the precursors and their cleavage products.

Five minutes after addition of AVP, processing of the precursors pVI and pVII was observed (**Fig. 1a** and **1b)**. For pVI, processing started with the appearance of the intermediate product corresponding to residues 33-250 (**Fig. 1b**), previously observed in *in vitro* assays using purified recombinant AVP and pVI (24). The mature protein, VI, appeared at t = 30 min, and became the dominant form after 1 hour of incubation, although some precursor was still present in the sample until t=5 hours. For pVII, precursor and mature forms coexisted for the first hour of AVP treatment, after which only the mature form was observed. It was not possible to determine when cleavage of pVIII started, because the available antibodies did not reveal its cleavage products. As with pVII, pVIII was completely processed after 1 h of AVP incubation, judging by the disappearance of the precursor band in western blots. For controls, treating intact *ts1* virus with AVP produced no cleavages, even after 6 h of incubation (**Supplementary Fig. 2**); neither did heat-disrupted *ts1* viruses incubated in the absence of AVP show any proteolytic degradation (see **Fig. 3b**).

L1 52/55k is a potential substrate for AVP, as it contains an AVP consensus cleavage sequence. Therefore, we probed disrupted *ts1* virus for this protein (**Fig. 1b**). Western blot analysis using serum against L1 52/55k revealed the presence of a band corresponding to the full length protein in *ts1* virus. After the addition of AVP, two more bands appeared beginning 15-30 min later, consistent with a 40 kDa doublet previously observed in (9) and (22). We named the bands in this doublet **F1a** and **F1b** (Fragment 1 a and b). A 40 kDa species is expected if the protein is cleaved at the consensus site. Consistent with this hypothesis, it has previously been shown that the 40 kDa doublet is not recognized by an antibody directed against the C-terminal region (residues 402-405) of L1 52/55k (22). Here we show directly that indeed the expected

cleavage is produced by AVP. An additional band of 34 kDa apparent molecular mass also appeared in the Western blots, that we call **F2**. This 34 kDa band recognized by anti-L1 52/55k antibodies had also previously been observed (9, 22), but this is the first direct proof that it originates from the proteolytic action of AVP. Furthermore, in gradient SDS-PAGE (**Fig. 1c**), at least three other bands recognized by the anti-L1 52/55k serum were observed upon incubation with AVP (**Fig. 1d**): two with apparent molecular masses larger than 30 kDa (**F3** and **F4**), and one of 18 kDa (**F5**). The presence of these bands hints at cleavage at non consensus sites. Interestingly, even after long incubations (t≥5 hours), a considerable amount of L1 52/55k in a variety of forms, from full length to 18 kDa, was observed, although the dominant form seemed to be the 40 kDa doublet bands. This is in contrast with the absence of L1 52/55k bands in wildtype, mature particles, suggesting that during maturation *in vivo* the processed forms of the L52/55k protein are somehow expelled, as opposed to them being completely degraded inside the nascent virion.

AVP cleaves L1 52/55k at non canonical sites

To further investigate the nature of the detected L1 52/55k species, we scanned the HAdV-2 L1 52/55k sequence (UniProt ID: P03262) for consensus AVP cleavage motifs using the PATTINPROT server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_pattinprot.html). There are three important positions in the AVP consensus cleavage sequences: P_4, P_2, and P_1-P_{-1} **(Table 1)**. When the search was restricted to 100% compliance to either of the two previously reported AVP recognition patterns (7, 8), only the consensus cleavage at residues Thr351-Gly352 was found, as expected. However, a search with relaxed pattern similarity constraints yielded 13 more possible cleavage sites (**Table 1**). According to the PATTINPROT similarity criteria, six sites at residues 36-37, 66-67, 353-354, 382-383, 384-385, and 398-399 had 75% similarity to the consensus patterns, with a wobble in the P_4 position. The other seven predicted sites had a lower similarity level (62%). Six of these (65-66, 71-72, 124-125, 275-276, 276-277, 300-301 and 380-381) had a wobble in the P_2 position, while the remaining one (297-301) had a wobble in the P_1-P_{-1} position. Location of these sites in the protein sequence (**Fig. 2a**) revealed that the 14

313 possible cleavages were clustered in 2 regions at the N-terminus, 3 at the C-terminus,
314 and 3 more in the central part of the polypeptide chain.

315 The possible L1 52/55k proteolytic products according to the PATTINPROT prediction
316 of AVP consensus and non-consensus cleavage sites are shown in **Table 2**.
317 Interestingly, there is a substantial correspondence between the sizes of the predicted
318 fragments and the bands recognized by anti-L1 52/55k antibodies in Western blots. To
319 further assess this point, these bands were extracted from gels and analyzed by mass
320 spectrometry (MS). The MS analysis confirmed that the F1, F2, F3 and F4 bands were
321 derived from L1 52/55k. It was not possible to determine the identity of the F5 band.
322 The peptide fingerprint obtained for each of the identified bands (**Fig. 2a**) further
323 supported their identity as fragments derived from non-consensus cleavage sites.
324 Altogether, our analyses indicate the following cleavage scheme for L1 52/55k (**Fig.**
325 **2b**): first, and most frequently, the C-terminal region is cleaved. The consensus
326 cleavage would produce a 40.3 kDa fragment, corresponding to the lower band in the F1
327 doublet. Cleavage at either the 382 or 398 positions (**Table 2**) would explain the upper
328 band F1a in the doublet, although there is no MS confirmation for this hypothesis. Next,
329 the 36 N-terminal residues are removed to produce F2 (36.3 kDa), in agreement with
330 previous results showing that this band did not appear in Western blots using antibodies
331 against either the C-terminal or the N-terminal regions of L1 52/55k, and that its
332 electrophoretic mobility was not dependent on the presence of reducing agents,
333 indicating that the single Cys residue at position 24 (**Fig. 2**) was absent (9, 22).
334 Additional cleavages identified here, at the 66 and 124 positions, give rise to F3 (33.0
335 kDa) and F4 (26.1 kDa). Finally, removal of the C-terminal stretch 275-351 or 300-351
336 could produce the smallest fragment observed, F5. It is intriguing that F5, whose
337 identity could not be confirmed by MS, consistently appears at early times during AVP
338 incubation (**Fig. 1c, 1d**).

339 Our results are consistent with L1 52/55k being cleaved at eight positions, seven of
340 them not conforming to the previously determined consensus sequence pattern (7, 8).
341 Other authors have previously detected maturation cleavages in adenovirus where the P_4
342 residue in the pattern was Gln or Asn, instead of Met, Leu or Ile (37). Here we report
343 one cleavage site with Asn in the P_4 position, other sites with Ala, Gly, and possibly
344 even a charged residue, Glu (**Fig. 2a**). The successive cleavages of L1 52/55k by AVP
345 result in fragments with drastic differences in their isoelectric points (**Table 2**), as well

as in the removal of sequence motifs or regions relevant for protein function (**Fig. 2b**). These include the only cysteine residue in the polypeptide chain; the two phosphorylation sites (38); part of the IVa2 binding domain (12); part of the region required for interaction with the viral packaging sequence (39); and the region containing residues mutated in the thermosensitive mutant *ts369* (16).

Cleavage of L1 52/55k by AVP requires the presence of dsDNA

We have previously shown that the presence of dsDNA is required for both activation of AVP and for cleavage of its substrates by AVP-pVIc complexes , when assayed in *ts1* virus particles completely disrupted by heating at 60ºC (24, 25). To see if this is also the case for L1 52/55k, we analyzed by Western blot its processing by AVP in the presence or absence of dsDNA (**Fig. 3**). As previously observed for other AVP substrates, when *ts1* virus was completely disrupted and its dsDNA digested away by DNase treatment (**Fig. 3a, lane 3**; and **Fig. 3b, lane 6**), no proteolytic products, including any processed form of L52/55k, were observed after 24 hours of incubation with AVP. When, after DNase treatment, the DNase was inactivated and purified *ts1* DNA was added back to the reaction, full AVP activity was recovered, including processing of L52/55k (**Fig. 3b, lanes 4 and 5**). Therefore, cleavage of L1 52/55k by AVP is also dependent on dsDNA.

An intriguing behavior was observed when instead of completely disrupting *ts1* by heating at 60ºC, we used milder disruption conditions, heating at 47ºC. In this case, treatment with DNase did not completely degrade the dsDNA, but instead fragments in the 200-300 bp size range remained in the solution, presumably protected by interactions with core proteins that are not disrupted in the whiffle ball particle (**Fig. 3a, lane 2**) (40). In the presence of these small dsDNA fragments, partial cleavage of L1 52/55k was observed, with only fragments F1 and F2 revealed in the Western blot (**Fig. 3b. lane 3**). That is, cleaving at internal positions in the polypeptide (interior to the 36-351 residue region) did not occur. In these conditions, digestion of the pVI, pVII and pVIII precursors was also deficient or absent (**Fig. 3b, SDS-PAGE**). This result indicates that AVP function is hindered by the lack of long stretches of dsDNA or by less DNA being present. It has previously been shown that for AVP to perform its function, both protease and substrate have to be bound to the same DNA molecule (24,

25). Therefore, a possible reason for this hindrance is the lack of physical space in the short oligonucleotides to house both the activated AVP and its substrates, as well as other DNA binding proteins. Another possible reason is that only those proteins with the lowest equilibrium dissociation constants for binding to DNA will be bound to the "small" amount of dsDNA left. In any case, the lack of minor, internal cleavages in L1 52/55k in a situation with limited access to dsDNA suggests that its interaction with the genome changes as a consequence of the major cleavages induced by AVP.

Cleavage of L1 52/55k impairs its interactions with other proteins in the viral particle

In the assays presented above, L1 52/55k protein was cleaved at multiple sites by the viral protease, but extensive cleavage was not observed – rather, large L1 52/55k fragments (over 17 kDa molecular weight) were present even after several hours of incubation with AVP. However, no traces of L1 52/55k were found in the mature virion in a variety of experiments (**Figs. 1b, 1d, 3b, 5**). Therefore, we sought to investigate how the limited proteolysis of L1 52/55k can result in complete removal of all fragments from the viral particle during *in vivo* assembly.

We have previously shown that *in vivo* maturation of adenovirus results in the removal of protein interactions that stabilize both capsid and core structures (4, 27). Here, we observe the same effect in our *in vitro* system. HAdV-2 *ts1* preparations were imaged by negative staining EM after heating at 47ºC during 10 minutes, and digestion with AVP for different periods. As expected, in the absence of AVP, *ts1* capsids lost some capsomers but largely retained their icosahedral organization. In contrast, after 45 min incubation with AVP, the structural integrity of the capsids was lost, and fragmented, flattened capsids were mainly observed, along with some unraveling cores (**Fig. 4a**). From this experiment we conclude that *in vitro* processing by AVP in disrupted immature virus particles weakens interactions stabilizing the viral particle, similar to the effect of *in vivo* AVP processing during maturation. Additionally, we observe that the multiple cleavages undergone by L1 52/55k result in fragments with different electrostatic properties (**Table 2**) and in loss of relevant sequence domains (**Fig. 2b**). These observations suggest that AVP-induced cleavages in L1 52/55k may diminish its

interactions with other viral proteins, or the viral DNA, facilitating its removal from viral particles during maturation.

Further evidence supporting this hypothesis was obtained from experiments testing the solubility of L1 52/55k before and after incubation with AVP (**Fig. 4b**). When heat disrupted *ts1* was centrifuged, the supernatant contained vertex proteins (peripentonal hexons, penton, IIIa, fiber, pVI), while cores (DNA, V, pVII) and presumably large capsid fragments (hexons, pVI, pVIII, IX) appeared in the sedimented material. Western blotting indicated that some L1 52/55k was released together with the vertices, but the majority remained in the insoluble fraction. When the disrupted virus was treated with AVP before centrifugation, the distribution of most viral components between supernatant and pellet remained essentially unaltered. However, all L1 52/55k fragments were solubilized, indicating that processing by AVP disrupts L1 52/55k interactions with the other proteins, and also the viral genome.

Next, we addressed the interactions of the different L1 52/55K fragments with other viral proteins using far Western blots (**Fig. 4c**). Disrupted *ts1* virus incubated with AVP was subjected to SDS-PAGE, L1 52/55k fragments F1, F2 and F3 were extracted and purified from gels, and labeled using either antibodies against L1 52/55k, or biotin. The labeled L1 52/55k fragments were then used as probes against viral proteins from *ts1*, processed or unprocessed by AVP. In all cases, the most intense signal was obtained with bands corresponding to either full length or processed L1 52/55k, indicating a strong tendency to form homooligomers. Additional, minor interactions were observed with hexon, penton base, pVII and VII. Interestingly, bands for these minor interactions became fainter as the L1 52/55k cleavages became more extensive. Interaction with penton base was only observed with F1, the largest fragment analyzed, while for the smallest (F3), virtually only self-interaction was observed. This result indicates that the N-terminal region of L1 52/55k (residues 1-65) participates in interactions with hexon, penton base and pVII/VII, but it is not required for self-interaction, in spite of containing the single cysteine residue in the sequence. Binding of L1 52/55k to hexon and penton base had not been previously described. The present analysis did not reveal other known L1 52/55k binding partners, such as polypeptides IIIa and IVa2 (12, 19). The signal for IVa2 may be obscured by the signal for L1 52/55k itself, since their electrophoretic mobilities are similar. As for IIIa, it is possible that the far Western blot refolding conditions did not produce the proper conformation to maintain the native

interactions. Nevertheless, the experiments presented here indicate that in the viral particle, L1 52/55k maintains an extensive network of interactions, not only with itself, but also with both shell (hexon, penton) and core proteins (pVII, VII), as well as with the genome. This network is disrupted when L1 52/55k is processed by AVP, providing a mechanism for scaffold ejection during maturation.

Estimation of the L1 52/55k copy number in immature *ts1* particles

In the course of both the present (**Fig. 1c**) and previous work (24), we noticed that bands appearing upon incubation of *ts1* with AVP, consistent with L1 52-55k cleavage products, were often visible by silver staining. This observation suggested the possibility that *ts1* full particles might contain L1 52-55k in larger quantities than expected from the copy number of 2 calculated in (9). Therefore, we decided to perform a new analysis of the L1 52/55k copy number in *ts1* using gel band densitometry (**Fig. 5**). Taking into account that *ts1* contains only full length L1 52/55k protein; that wt virions do not contain L1 52/55k; and that full length L1 52/55k and polypeptide V have similar electrophoretic mobility (~50 kDa), we estimated the amount of L1 52/55k in *ts1* by comparing the density ratio between the 50 kDa band and other bands corresponding to proteins with known copy numbers and present in both viral species. The excess protein content in the *ts1* 50 kDa band was attributed to L1 52/55k (**Table 3**). These calculations indicated that immature particles contain more than 50 copies of L1 52/55k, an order of magnitude more than previously reported (9).

Discussion

Adenovirus morphogenesis is a complex process involving a considerable number of players. The final components of the HAdV-2 ~150 MDa mature particle include multiple copies of seven different proteins in the icosahedral shell (hexon, penton base, fiber, IIIa, VI, VIII, and IX), and three putative DNA condensing proteins bound to the genome in the core (V, VII and μ) (41, 42). In addition, several other factors are required to assemble the fully infectious particle. The concerted action of at least five different proteins is required for successful genome packaging: IIIa (also a part of the icosahedral shell), IVa2, L4 33k, L4 22k and L1 52/55k (10, 12-15, 20, 43). Finally, proteolytic maturation carried out by AVP together with its cofactors (dsDNA and pVI_C) is required to render the particle infectious, i.e. metastable and primed for uncoating (2, 4, 23).

Here we have followed the processing of immature, fully packaged virus particles using recombinant AVP. We have observed that in this *quasi-in vivo* system, processing of polypeptide VI proceeds in two steps, from the precursor pVI to the intermediate iVI to the mature form VI, as previously observed using purified, recombinant pVI (24). However, the most novel information provided by this work links the genome encapsidation and maturation processes, by showing that L1 52/55k is the seventh substrate for AVP, and pointing to the effect of the AVP-induced cleavages on its extrusion from the maturing particle.

Because L1 52/55k is easily detected in HAdV light density particles (considered assembly intermediates) but is absent from the mature virion, it has been proposed to act as a scaffolding protein (9). Scaffolding elements are crucial in the assembly of large, complex capsids, and are best understood in the dsDNA bacteriophage and the structurally related herpesvirus systems (44-46). Some of their functions include: initiating assembly by nucleation of coat proteins; determining capsid size; and stabilizing labile assembly intermediates. Although structural knowledge on scaffolding proteins is scarce, studies on bacteriophage assembly indicate that they have a tendency to form homo-oligomers; bind to major coat proteins and packaging machinery (47, 48); and have non-specific dsDNA binding activity (49). Once their functions have been fulfilled, scaffolding elements are released from the immature capsid, before or during genome packaging. Release is often mediated by degradation by viral proteases and is

thought to occur by extrusion through channels in the immature shell. In some bacteriophage, such as HK97, there are no separate scaffolding polypeptides. Instead, specific domains in the coat protein aid in assembly initiation and elongation and are proteolytically removed before packaging.

Adenovirus seems to use a dual scaffolding system, including both a detached polypeptide (L1 52/55k) and flexible regions of minor coat proteins that are removed during maturation (27, 41). We have previously shown that the immature *ts1* particle is more stable than the mature one, largely due to a remarkable core compaction and the presence of strong capsid-core interactions mediated by the pIIIa, pVI, pVII, pVIII and pre-μ precursors (4, 5, 27). In these studies the presence of L1 52/55k was not considered, as a very low copy number (n=2) had been reported in *ts1* full particles (9). However, the results shown here indicate that there is a much larger quantity of L1 52/55k (n=50) in *ts1*, implying that L1 52/55k may also contribute to the higher stability of the immature virus. Here we show for the first time that L1 52/55k is proteolytically processed by AVP, and that this processing facilitates its removal from the assembled particle by impairing interactions with other core and shell proteins and possibly with the dsDNA genome itself. Thus, cleavage of L1 52/55k must be considered part of the maturation process of adenovirus during which it is primed for uncoating.

Why our estimates for the copy number of the L1 52/55k protein in *ts1* virus differ from previous reports is not clear. The highly dynamic and transitory nature of the L1 52/55k protein in the virus makes its copy number difficult to measure. Several potential problems could give rise to this disagreement. First, the amount of viral DNA per virion, as maturation proceeds, is variable. Second, in the growth of *ts1* virus, slight, local variations in temperature could allow some AVP to be packaged which would decrease the amount of L1 52/55k protein. Finally, quantitating proteins by staining or antibody reactivity can give variable results. In the future, other methods for quantitating the copy number of L1 52/55k in *ts1* particles may help to settle this discrepancy. For example, metabolic labeling by propagating the virus in the presence of ^{35}S provided a very accurate stoichiometric analysis of the complete AdV mature particle (50). However, the occurrence of polypeptides IVa2, V and L1 52/55k in a narrow range of electrophoretic mobility may complicate the procedure. An alternative method would be the use of quantitative mass spectrometry (51). For these studies, representative tryptic peptides for the protein under study are synthesized, isotopically

529 labeled and mixed in known quantities with the viral sample. The mass spectrometry
530 signal intensities of the labeled peptides are compared with those of their unlabeled
531 partners, thus serving as internal controls for estimation of the abundance of the
532 corresponding unlabeled peptide in the virus. This methodology has also been used for
533 AdV, where it helped to determine the copy number of the low abundance polypeptide
534 IVa2 (52).

535 Incubation with recombinant AVP produced L1 52/55k fragments different from those
536 expected if the protein was cleaved at the single AVP consensus pattern in its sequence
537 (7). The observation of cleavages at sites not fully conforming to the consensus was not
538 that surprising, given that this phenomenon had already been observed (37, 53). The
539 four empirically identified fragments shown by MS to be derived from the L1 52/55k
540 protein were all derived by cleavage at the AVP consensus sequence near the C-
541 terminus, at position 351. Three of these fragments were also derived by a second
542 cleavage, near the N-terminus of L1 52/55k, at sites that resemble consensus cleavage
543 sites (**Fig. 2** and **Table 1**). In fact, all of the putative cleavage sites resemble AVP
544 consensus cleavage sites; they all contain 2 of the 3 determinants most preferred by the
545 proteinase. This observation argues that cleavage occurred by AVP and not by a
546 contaminating *E. coli* protease in the AVP preparation. Consistent with this conclusion
547 is that no cleavage by AVP occurred in disrupted virions treated with DNase (**Fig. 3**).
548 No *E. coli* protease is known to have a DNA-dependent activity, whereas DNA is a
549 cofactor for AVP activity (23-25). Since three of the four fragments were cleaved at the
550 AVP consensus sequence as well as a secondary site, it is possible that cleavage at the
551 consensus sequence resulted in a conformational change of the protein thereby exposing
552 several secondary cleavage sites. Indeed, one could hypothesize that this first
553 conformational change is the trigger for a cascade of cleavages required for release of
554 L1 52/55k fragments from the nascent virion.

555 The picture emerging from this and other work highlights the similarities and the
556 differences between adenovirus and bacteriophage/herpesvirus regarding assembly and
557 maturation. Adenovirus scaffolding elements, whether peptides attached to minor coat
558 proteins or polypeptide L1 52/55k, form part of an extensive network of interactions
559 between the icosahedral shell and the viral core. Like the phage P22, □29 or SPP1
560 scaffolding proteins (45), L1 52/55k has the ability to form homo-oligomers, either
561 mediated by sulfur bridges using its single cysteine (9) or not (**Fig. 4c**). Additionally, L1

52/55k interacts with coat and core proteins (**Fig. 4c**) (12, 21), as well as with other elements of the packaging machinery (17), including the genome itself (21). The latter property is a requisite for its functioning as an AVP substrate (**Fig. 3**) (25). L1 52/55k is cleaved at multiple sites, including several non-consensus ones, by the viral protease (**Fig. 2**), but the protein is not completely degraded (**Fig. 1**). Cleavages impair some of its interactions, leading to release of L1 52/55k from the maturing particle, similar to the release mechanism reported for herpesvirus (46). The exact nature of the altered interactions is not known. However, it is noticeable that the different L1 52/55k fragments have widely different charge values (**Table 1**), suggesting possible changes in electrostatic interactions. Interestingly, in bacteriophage P22, electrostatic interactions between scaffold and coat proteins have been directly observed in high resolution cryo-EM studies (47).

In adenovirus, the existence of light density, empty or partially packaged capsids containing immature protein precursors, together with the similarities between polypeptide IVa2 and other packaging motors, point to a sequential packaging mechanism similar to that of bacteriophage, with the dsDNA being pumped into a preformed procapsid (13, 17, 43, 54-56). Unlike in bacteriophage, in adenovirus the scaffolding protein L1 52/55k does not seem to be required for capsid assembly, nor for incorporation of the packaging motor (10). Rather, L1 52/55k participates in bridging the viral core to the shell (**Fig. 6**). A possible role for this protein during packaging would then be to tether stably the partially packaged genome to the procapsid while DNA pumping is taking place. Alternatively, in a concerted assembly and packaging model, the role of L1 52/55k could be to recruit capsid proteins, or capsid fragments, onto condensing core precursors. In any case, since binding to DNA of both AVP and its substrates is required for proteolysis to occur (24, 25), scaffold maturation and release must occur during or after genome encapsidation.

How are the large cleavage products from L1 52/55k ejected from the virus particle? The final products of L1 52/55k proteolytic processing are rather large polypeptides, ranging in size from 17 to 40 kDa. Cleavage and release of L1 52/55k is not a requirement for successful packaging, since immature virus particles containing full length L1 52/55k and genome are readily assembled in the *ts1* mutant. The immature capsids of bacteriophage and herpesvirus have large openings (~4x2 nm), thought to be used as extruding conduits for the scaffold proteins (45). These channels disappear due

595 to large conformational rearrangements occurring during maturation and packaging to
596 produce a tightly closed shell. Such a mechanism does not seem to exist in adenovirus.
597 Light density particles in CsCl gradients are thought to represent the adenovirus
598 procapsid, but their structure has not been solved. However, negative staining EM
599 images, for example those in (13), suggest that their gross organization is highly similar
600 to that of the mature virion. Empty and full capsids of the adenovirus-like bacteriophage
601 PRD1 have practically identical structures, as do the adenovirus mature and immature,
602 fully packaged viral particles (27, 57). Therefore, in adenovirus, building of the tightly
603 knit immature shell must proceed in such a way that a large opening is present until the
604 last stages of maturation, so that scaffold fragments can be released before building of
605 the icosahedral shell is completed. Once core and capsid precursors are brought
606 together, whether in a sequential or concerted manner, the DNA-bound AVP can be
607 activated by interaction with pVI_C; maturation and packaging would proceed in a
608 simultaneous fashion, and assembly would end by fitting a last capsid piece after
609 complete release of scaffold fragments. What this last capsid piece would be, and
610 whether the large opening matches the genome packaging channel, are questions that
611 remain to be answered.

612

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Figure legends

Figure 1. Time course of AVP activity on mildly disrupted *ts1 virus*. (a) A 15% acrylamide SDS-PAGE showing changes in the viral proteins upon incubation with AVP for the times indicated. **(b)** Western blot assays to follow proteolytic processing of pVI, pVII, pVIII and L1 52/55k, as indicated. **(c)** Time course analysis of AVP cleavages in a 4-20% gradient gel. Double stars indicate bands generated upon incubation with AVP that could correspond to L1 52/55k cleavage products. **(d)** Western blot for L1 52/55k protein on a 4-20% gradient gel reveals more AVP generated proteolysis products. L1 52/55k full length precursor (**FL**) and fragment products (**F1** to **F5**) are indicated in (b) and (d). **(e)** Control experiment. No proteolytic processing is observed in intact *ts1* virions in the presence of AVP, even after 6 hours of incubation. This observation also indicates that the virus particle is sealed, at least to a protein the size of AVP.

Figure 2. Cleavage pattern of L1 52/55k by AVP. (a) Location in the L1 52/55k sequence of the predicted AVP cleavage motifs and of the actual peptides identified by MS/MS in the different fragments generated by AVP cleavage. The possible cleavage sites predicted by PATTINPROT are indicated by vertical red lines. A thick, continuous line indicates the consensus site. Thick dashed lines indicate sites with 75% similarity to the consensus pattern. Thin dashed lines indicate sites with 62% similarity to the consensus pattern. The peptides identified for each fragment are indicated by horizontal lines below the sequence in different colors: blue for F1, orange for F2, green for F3 and magenta for F4. The single Cys residue is highlighted in green and the two phosphorylated Ser residues in light blue. **(b)** Schematics picturing the L1 52/55k sequence and cleavage products observed in this work. The box at the top represents the full length sequence with some relevant motifs or regions indicated as follows: pink, domain required for genome packaging (39); orange, IVa2 interacting domain (12); green, Cys residue involved in homodimer formation; blue, phosphorylated Ser residues; and dark red, residues involved in the *ts369* mutation (16). The fragment assignment and molecular weight are indicated in the center of each box. Initial and

final positions in the L1 52/55k sequence are indicated at left and right of each box. Stars indicate fragments identified by MS/MS.

Figure 3. DNA requirement for L1 52/55k cleavage by AVP. (a) DNA present in the preparations. DNA was extracted from either intact or heat disrupted *ts1* virus particles after DNase treatment, and revealed by Southern blot with a viral genome probe. Lane 1: intact virus. Lane 2: virus disrupted at 47ºC. Lane 3: virus disrupted at 60ºC. **(b)** SDS-PAGE (top) and Western blot for L1 52/55k (bottom) showing dependence on DNA of AVP activity. Lane 1: control wildtype virus. Lane 2: control, intact untreated *ts1* virus. Lane 3: *ts1* virus disrupted at 47ºC, DNase treated and then incubated with AVP. Lane 4: *ts1* virus disrupted at 60ºC, treated with DNase, DNase inactivated, and purified DNA added back before incubation with AVP. Lane 5: *ts1* virus disrupted at 60ºC and incubated with AVP. Lane 6: *ts1* virus disrupted at 60ºC, treated with DNase and incubated with AVP. Lane 7: *ts1* virus disrupted at 60ºC, incubated with buffer in the absence of AVP.

Figure 4. Effect of L1 52/55k cleavage in L1 52/55k associations with other components of the viral particle. (a) EM images showing additional disruption of *ts1* particles heated at 47ºC upon incubation with AVP for 45 min. The inset shows an unraveling core. The bar represents 200 nm. **(b)** Solubility of L1 52/55k and its cleavage products. Mildly disrupted *ts1* virus, control or treated with AVP, was centrifuged. The input (**I**), supernatant (**S**) and pellet (**P**) were analyzed by native DNA electrophoresis, SDS-PAGE, and Western blot with antibodies against fiber, L1 52/55k, or polypeptide V, as indicated. **(c)** Far Western blot assays to analyze interactions between L1 52/55k cleavage products and other proteins in the viral particle. The proteins in control or AVP treated *ts1* virus samples were separated by SDS-PAGE, and probed with gel-purified L1 52/55k F1, F2 and F3 fragments, previously labeled with either antibodies against L1 52/55k or biotin, as indicated.

Figure 5. Estimation of L1 52/55k copy number. Serial dilutions of wildtype (**wt**) or *ts1* virus samples were analyzed by SDS-PAGE and band densitometry, as well as by Western blot for L1 52/55k or polypeptide V, as indicated. The intensity ratio between

855 the bands labeled in the SDS_PAGE panel was used for the estimation, as explained in
856 the text.

857 **Figure 6. Cartoon summarizing the multiple interactions established by L1 52/55k**
858 **in the viral particle.** The figure is based both in results presented here and in
859 previously published studies (see text for details).

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862 **Tables**

863

864 **Table 1:** Potential AVP cleavage sites in L52/55k predicted by PATTINPROT.
865 Discrepancies from the consensus patterns are in **bold**.
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Position relative to cleavage site (--)	$P_4P_3P_2P_1$--P_{-1}
AVP consensus cleavage sequences	(M/I/L)XGX--G (M/I/L)XGG--X
Major cleavage sequence in the L1 52/55k protein	LAGT--G
Wobble in the P_4 position	**A**SGG--A **E**EGE--G **G**TGS--G **A**AGA--G **G**AGP--G **N**VGG--V
Wobble in the P_2 position	LE**E**G--E LA**R**L--G LR**H**G--L ML**S**L--G LS**L**G--K LE**A**A--G
Wobble in the P_1-P_{-1} position	IEG**F**--**Y**

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869 **Table 2:** Possible L1 52/55k fragments derived from AVP cleavage at the
870 PATTINPROT predicted sites, and their correspondence to bands observed in Western
871 blots after *in vitro* processing of mildly disrupted *ts1* virus by AVP. The isoelectric
872 point value for each fragment is indicated. A star (*) indicates identification by mass
873 spectrometry.

Fragment	Calculated molecular weight (kDa)	pI	Possible correspondence to Western blot band
1-415 (full length)	46.9	5.67	Full length
1-398	44.9	7.24	F1a
1-382	43.5	6.89	F1a
1-351 (consensus)	40.3	7.92	F1b*
36-351	36.3	6.66	F2*
66-351	33.0	8.77	F3*
124-351	26.1	6.74	F4*
124-300	20.2	5.55	F5
124-275	17.2	5.04	F5
275-351	8.9	9.91	Not observed

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Table 3: Estimation of L1 52/55k copy number in *ts1*virus

	wt virus		*ts1*virus		Deduced L1 52/55k copy number in *ts1* virus (3)
	Theoretical mass ratio (1)	Calculated mass ratio (2)	Theoretical mass ratio (1)	Calculated mass ratio (2)	
VII/V	2.84	2.64 ± 0.20	2.50	2.12 ± 0.09	53 ± 5
IX/V	0.53	0.51 ± 0.04	0.53	0.34 ± 0.03	55 ± 3

(1) Calculated from the corresponding protein molecular mass (*M*) and copy numbers (*N*) as follows: in wt, $ratio_{VII/V} \approx (M_{VII}*N_{VII})/(M_V*N_V)$; in *ts1*, $ratio_{pVII/V} \approx (M_{pVII}*N_{pVII})/(M_V*N_V)$; in both, $ratio_{IX/V} \approx (M_{IX}*N_{IX})/(M_V*N_V)$. The following values were used: M_V = 41.0 kDa; M_{pVII} = 22.0 kDa; M_{VII} = 19.4 kDa; M_{IX} = 14.3 kDa; N_V = 157; $N_{pVII} = N_{VII}$= 833; N_{IX} = 240.

(2) Calculated from the optical density values (*D*) of gel bands as measured with ImageJ, for example: $ratio_{VII/V} \approx D_{VII}/D_V$. The results report the average and standard deviation values from three independent electrophoresis assays.

(3) Calculated by solving the following equations for N_{L1}: $ratio_{pVII/(V+L1)} \approx D_{pVII}/D_{(V+L1)} \approx (M_{pVII}*N_{pVII})/(M_V*N_V+M_{L1}*N_{L1})$; and $ratio_{IX/(V+L1)} \approx D_{IX}/D_{(V+L1)} \approx (M_{IX}*N_{IX})/(M_V*N_V+M_{L1}*N_{L1})$, with M_{L1} = 47.0 kDa.