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Chaperone-Mediated In Vitro Disassembly of Polyoma- and Papillomaviruses

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Hsp70 chaperones play a role in polyoma- and papillomavirus assembly, as evidenced by their interaction in vivo with polyomavirus capsid proteins at late times after virus infection and by their ability to assemble viral capsomeres into capsids in vitro. We studied whether Hsp70 chaperones might also participate in the uncoating reaction. In vivo, Hsp70 coimmunoprecipitated with polyomavirus virion VP1 at 3 h after infection of mouse cells. In vitro, prokaryotic and eukaryotic Hsp70 chaperones efficiently disassembled polyoma- and papillomavirus-like particles and virions in energy-dependent reactions. These observations support a role for cell chaperones in the disassembly of these viruses.

The *Papillomaviridae* and *Polyomaviridae* are small, nonenveloped DNA viruses that have structurally similar 50- to 55-nm-diameter capsids comprised of 72 capsomeres arranged in a T = 7 icosahedral lattice (2). Each capsomere is a pentamer of the major capsid protein: L1 for papillomavirus and VP1 for polyomavirus. The carboxy terminus of L1 or VP1 mediates interpentameric contacts in the assembled capsid (13, 16, 17, 22). These contacts are stabilized by disulfide bonds for papillomaviruses (15, 21, 29) or by both disulfide bonds and calcium bridges for polyomaviruses (4, 5, 14).

Some papillomaviruses enter the cell via a slow version of clathrin-dependent receptor-mediated endocytosis (3, 9, 10, 31, 32), and disassembly may be initiated in late endosomes before escape into the cytoplasm (9). Polyomavirus entry pathways converge on the endoplasmic reticulum (ER), where disassembly and release of a dissociated viral nucleoprotein complex into the cytosol may occur (25, 26). Disassembly of simian virus 40 begins within 5 h postinfection (hpi) in the ER (25); murine polyomavirus VP1 colocalizes with the ER resident Hsp70 family chaperone BiP by 3 hpi and subsequently can be detected in perinuclear regions (19, 27). While there is no evidence for Hsc70/BiP functioning in the uncoating of polyoma- and papillomaviruses, previous studies have demonstrated an interaction between Hsp70 family chaperones and polyomavirus VP1 late in infection during virion assembly (1, 8), and Hsc70-mediated in vitro assembly of both polyoma- and papillomavirus capsids from VP1 and L1 has been observed (7; L. R. Chromy and R. L. Garcea, unpublished data).

Observations during the study of in vitro chaperone-mediated assembly of capsomeres suggested that the chaperone reaction may be reversible (Chromy and Garcea, unpublished data). Therefore, we hypothesized that Hsp70 family chaperones may also participate in the disassembly of entering polyoma- and papillomavirus virions.

Hsp70 chaperones associate with polyomavirus virions early in infection. To determine whether cellular Hsp70 chaperones associate with incoming virions during infection, coimmunoprecipitation experiments were performed with murine polyomavirus-infected NIH 3T3 cells. Uninfected cells or cells at 1, 2, or 3 hpi were harvested in radioimmunoprecipitation assay buffer containing 10 mM ADP to inhibit dissociation of chaperones (7, 8). The lysate was coimmunoprecipitated with either I58, an anti-VP1 rabbit antibody, or SPA820, an anti-Hsp/Hsc70 mouse antibody (Stressgen). The converse antibody was used to detect the interacting protein by immunoblotting.

As seen in Fig. 1, an association between Hsc70 and VP1 was detected in both immunoprecipitation reactions at 3 hpi, although the interaction was more apparent with the anti-Hsc70 antibody (Fig. 1B). Hsc70 binding to VP1 was not detected

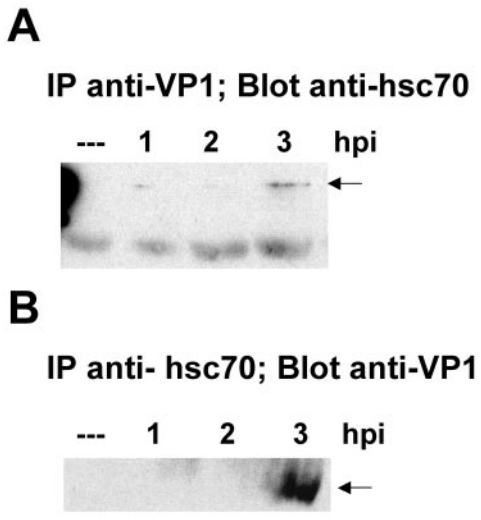

FIG. 1. Association of Hsc70 with polyomavirus virion VP1 during virus entry. Murine polyomavirus-infected NIH 3T3 cells were harvested at 1, 2, or 3 hpi in radioimmunoprecipitation assay buffer containing 10 mM ADP. The lysate was immunoprecipitated and analyzed by Western blotting using the indicated antibodies. Arrows indicate bands of interest.

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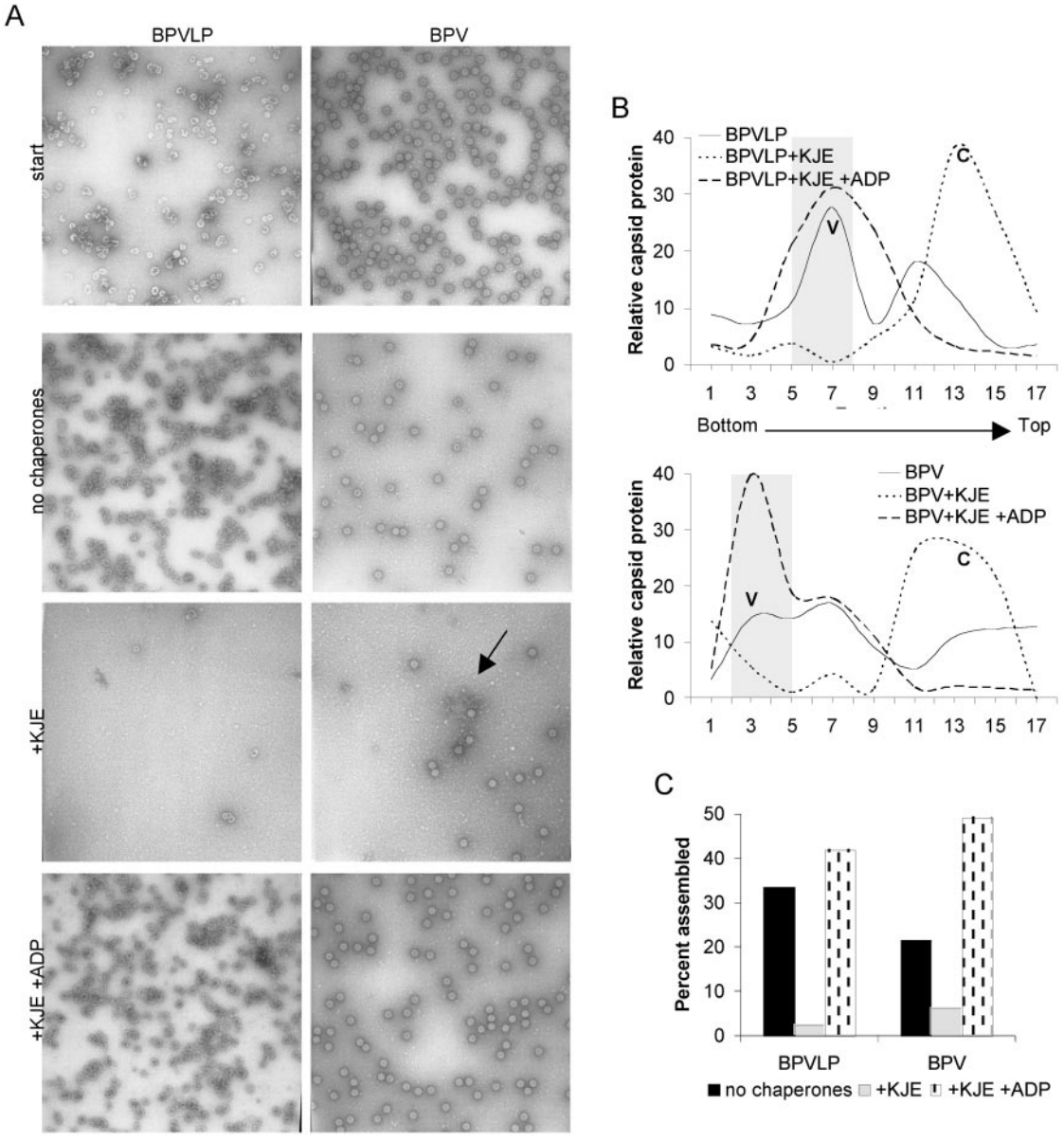

FIG. 2. Prokaryotic chaperones disassemble BPV virions and VLPs (BPVLP). In vitro reactions were performed by dialysis overnight against disassembly buffer (no dithiothreitol, with calcium and ATP or ADP where indicated). (A) TEM showing disassembled particles (arrow) in samples with chaperones (+KJE). (B) Reactions analyzed by sucrose gradient sedimentation. V, assembled particles; C, capsomere controls. (C) Quantitation of the assembled fractions (shaded in panel B).

after 3 hpi (not shown). Although supportive of an interaction between virions and Hsp70 during uncoating, the analysis is difficult because of the relatively low numbers of infecting virions and thus a small amount of VP1. Therefore, we continued to study the interaction of cellular chaperone proteins with both polyoma- and papillomavirus by using in vitro biochemical assays.

Disassembly of virions and VLPs by prokaryotic chaperones. Recombinant bovine papillomavirus (BPV) L1 virus-like particles (VLPs) and BPV virions isolated from cow warts (15) were purified by density ultracentrifugation in CsCl. CsCl was removed by dialysis into gradient buffer (20 mM Tris-Cl [pH 7.2], 5% glycerol, 180 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$), and the VLPs or virions were then treated with either no chaperones or the purified recombinant prokaryotic chaperones DnaK, DnaJ, and GrpE (KJE) (Stressgen) (7). These reactions were carried out by dialysis against reaction buffer (50 mM Tris-Cl [pH 7.2], 5% glycerol, 180 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 20 mM $MgCl_2$, 20 mM ATP) or against reaction buffer with 20 mM ADP instead of ATP overnight at room temperature with 4 μM KJE.

Reaction mixtures were absorbed to glow-discharged, carbon, and Formvar-coated copper grids (G400 copper; EM Sciences), rinsed with buffer lacking phosphates, stained with uranyl acetate, and examined by transmission electron microscopy (TEM) (Philips CM10) at 80 kV. No morphological differences

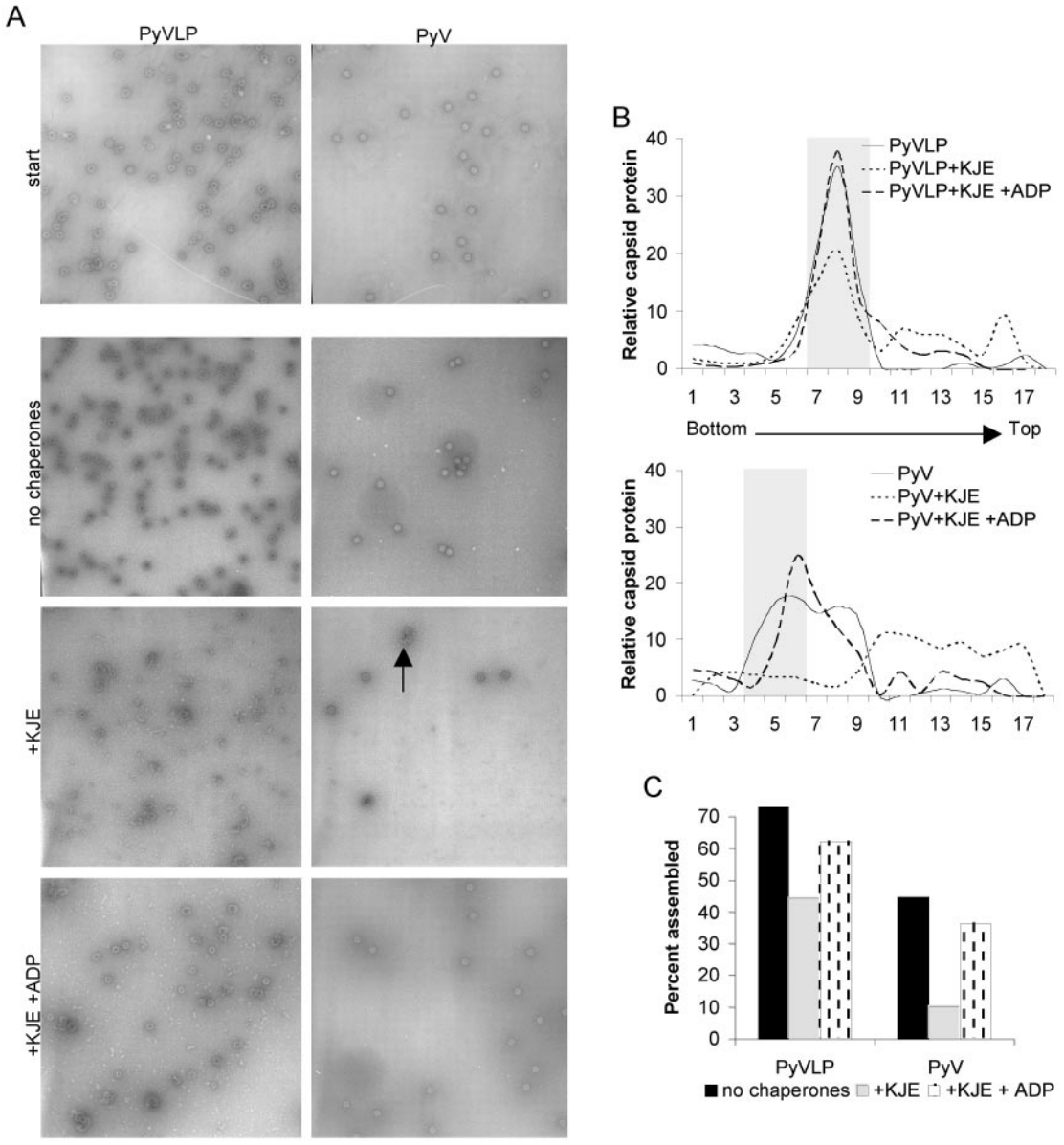

FIG. 3. Prokaryotic chaperones disassemble murine polyomavirus virions (PyV) and VLPs (PyVLP). In vitro reactions were performed by dialysis overnight against disassembly buffer (no dithiothreitol, with calcium and ATP or ADP where indicated). (A) TEM showing disassembled particles (arrow) in samples with chaperones (+KJE). (B) Reactions analyzed by sucrose gradient sedimentation. (C) Quantitation of the assembled fractions (shaded in panel B).

between the starting samples in CsCl and the dialyzed particles were seen (Fig. 2A). The addition of prokaryotic chaperones resulted in a decreased number of assembled particles per field and a concomitant increase in the amount of pentamers and background amorphous material (Fig. 2A). In the BPV-plus-KJE samples, capsid proteins may be present as aggregates on the viral genome (Fig. 2A, arrow). No disassembly was observed when chaperone activity was inhibited by excess ADP (Fig. 2A).

The products of the in vitro reactions were analyzed by sucrose gradient sedimentation to separate VLPs or virions from the disassembly intermediates and to quantify the extent of disassembly. Ten to 50% discontinuous sucrose gradients were prepared with gradient buffer (20 mM Tris-Cl [pH 7.2], 5% glycerol, 180 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) in 2.2-ml tubes (Beckman). Samples were sedimented in an RP55S-351 swinging bucket rotor in a Sorvall RC M120 ultracentrifuge at 50,000 rpm for 30 min at 4°C. Fractions were trichloroacetic acid precipitated, and capsid proteins were assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gradients demonstrated that the assembled particles seen in the samples without chaperones were shifted to slower-sedimenting products in the reactions with chaperones (Fig. 2B). The products from the BPV VLP-plus-KJE reactions sedimented at the position of capsomere controls, while the BPV-plus-KJE reactions had a wider sedimentation pro-

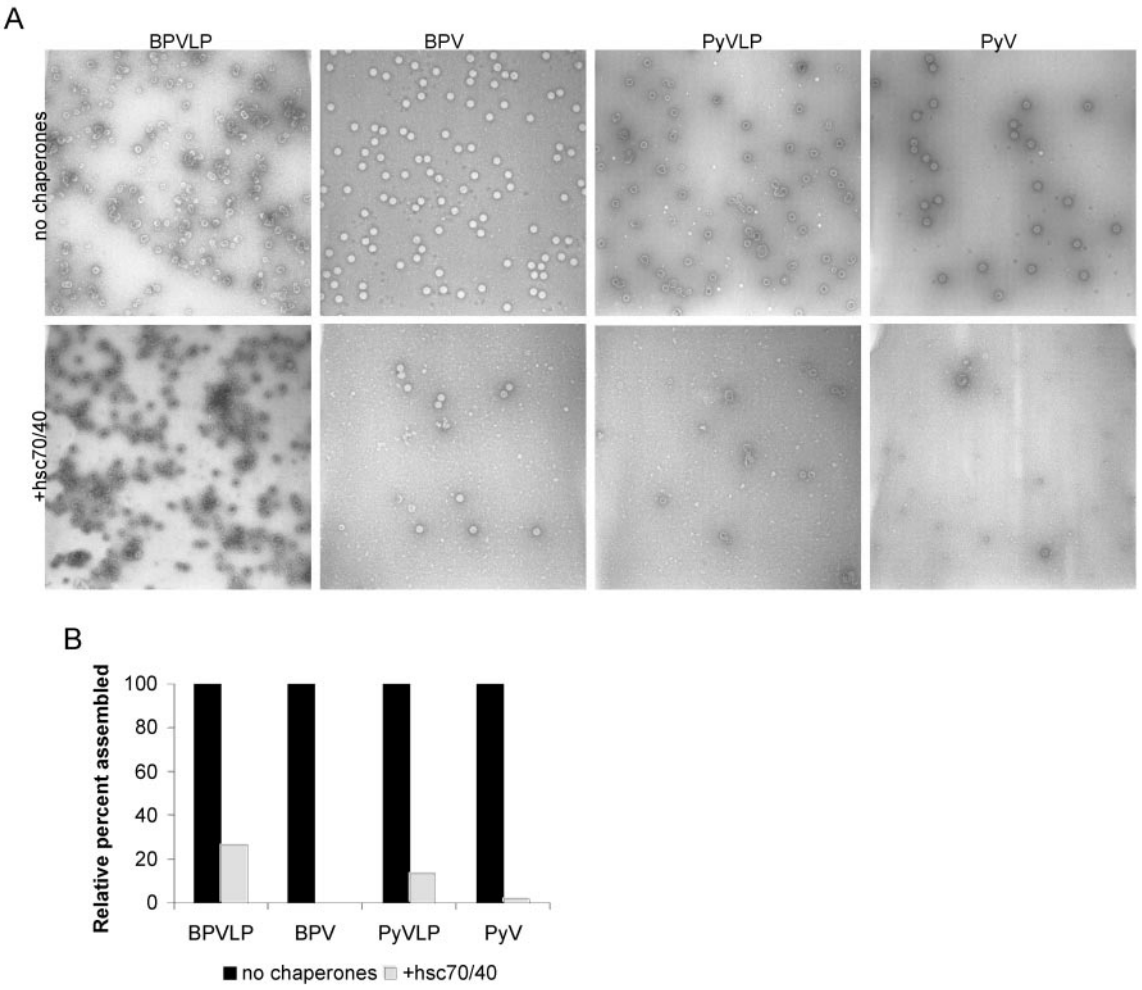

FIG. 4. Eukaryotic chaperones disassemble papillomavirus (BPV) and polyomavirus (Py) virions and VLPs. In vitro reactions were performed by overnight dialysis against disassembly buffer. (A) TEM of samples with Hsc70 and Hsp40 chaperones (+hsc70/40). (B) Quantitation of fractions analyzed by sucrose gradient sedimentation.

file, consistent with the genome-plus-capsid-protein aggregates seen by TEM. The presence of ADP inhibited chaperone disassembly of particles and also appeared to stabilize the inherent background dissociation seen in samples without chaperones. A similar stabilization was seen for polyomavirus particles, but to a lesser extent (see below). The assembled particles (Fig. 2B, shaded regions) were quantitated (Fig. 2C), and the number of VLPs or virions was significantly reduced by chaperone-mediated disassembly in an energy-dependent manner. The relatively low percentage of assembled particles in the no-chaperone samples may represent a compromise between the need to stabilize assembled particles and the optimization of buffer conditions for chaperone activity. All of the disassembly reactions were repeated multiple times with similar results; however, only the data from the optimized buffer conditions are shown.

In vitro disassembly reactions were also performed with recombinant polyomavirus VLPs or polyomavirus virions purified, respectively, from recombinant baculovirus-infected Sf9 cells or virus-infected NIH 3T3 cells (12, 30). In vitro disassembly reactions were carried out as described for papillomavirus VLPs and virions. TEM demonstrated disassembly in the presence of prokaryotic chaperones, and the chaperone-mediated disassembly was inhibited by ADP (Fig. 3A). Again, capsid proteins appeared to form aggregates with the viral genome in the virion-plus-KJE samples (Fig. 3A, arrow).

The products of the polyomavirus reactions were analyzed by sucrose gradient sedimentation (Fig. 3B). The polyomavirus virion sedimentation profile, although broadened in this experiment, was similar to that observed for BPV, with a shift to slower-sedimenting products in the presence of chaperones that was inhibited by ADP. However, the polyomavirus VLP reactions did not demonstrate disassembly as complete as that seen for BPV VLPs. Polyomavirus VLP disassembly may have been partially inhibited by calcium in the reaction buffer, which both stabilizes and promotes VLP assembly. Additionally, chaperones assemble VP1 capsomeres into capsids in the presence of ATP, although very few of these assemblies were identified. Reassembly probably did not occur in the polyomavirus virion disassembly reactions because the dissociated capsomeres

likely formed aggregates with the viral minichromosomes. Nevertheless, quantitation of the assembled fractions revealed chaperone-mediated disassembly that was energy dependent (Fig. 3C).

Previous studies have demonstrated that disulfide bond-reducing agents expand BPV (15, 21, 29) and that disulfide bonds and calcium bridges stabilize polyomavirus virions (4, 5, 14). However, chelating and reducing agents did not enhance chaperone-mediated disassembly of either papilloma- or polyomavirus virions (data not shown).

Eukaryotic chaperone-mediated disassembly of polyoma- and papillomavirus VLPs and virions. We also determined whether eukaryotic chaperones could disassemble polyoma- and papillomavirus capsids in the same manner as did the prokaryotic factors. Disassembly reactions were carried out with BPV VLPs, BPV virions, polyomavirus VLPs, and polyomavirus virions with or without addition of the eukaryotic chaperones Hsc70 and Hsp40 (at 4 μM and 0.4 μM, respectively). As in the TEM analysis of the previous experiments, the samples with chaperones showed disassembly products (Fig. 4A). The BPV VLP-plus-Hsc70/40 reaction had residual assembled products, and these observations were consistent over several experiments. Sucrose gradient analysis confirmed disassembly by chaperones, including the disassembly of BPV VLPs, as evidenced by a shift to slower-sedimenting fractions (quantitation in Fig. 4B).

Discussion. Chaperones assist in many stages of virus life cycles (20) and have been implicated in the disassembly of adenovirus (6, 23, 28). Our previous studies identified a role for Hsp70 chaperones in the late stages of infection, when virions are being assembled (7, 8). The current results show that Hsp70 chaperones can disassemble viral capsids in the reverse reaction. The direction of the equilibrium could depend on cochaperones found in particular cellular compartments or other regulatory factors (reviewed in reference 35).

Capsid dissociation is likely initiated before the viral genome enters the cytosol, so that the genome will be competent for nuclear import. Polyoma- and papillomavirus virions must undergo considerable conformational changes during disassembly, since they are too large (50 to 55 nm) to be transported intact through the nuclear pore, (reviewed in reference 33). Although we found that significant morphological and structural changes resulted from chaperone action, the observed virus disassembly intermediates may not be completely "uncoated." Complete removal of capsid proteins may be disadvantageous, since the viral genome likely could not be imported to the nucleus without the karyophilic signals of bound capsid proteins, as shown for the nuclear localization signal of polyomavirus VP3 (17).

The entry pathway is critical for initiating disassembly and raises interesting paradoxes for these viruses, given the bonds stabilizing their capsids. For example, the calcium concentration is high and the disulfide reducing capacity is relatively low in the ER. Thus, the uncoating reaction must prevail despite unfavorable environmental conditions. Indeed, we detected little to no enhancement of chaperone-mediated disassembly in the presence of chelating and reducing agents. Cofactors in vivo may modulate the unfavorable environmental conditions. For example, thiol-redox reactions are mediated by the protein disulfide isomerase (PDI) family, and these proteins can be found at the cell surface through the ER. Some PDIs contain J domains (reviewed in reference 11), suggesting that PDIs may recruit and stimulate Hsp70 chaperones or have intrinsic chaperone activity (24, 34), and recently the PDI-like protein Erp29 was shown to mediate polyomavirus membrane penetration (18). Our observations support a step in which Hsc70-like chaperones continue the uncoating process.

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REFERENCES

1. **Becker, K. A., L. Florin, C. Sapp, G. G. Maul, and M. Sapp.** 2004. Nuclear localization but not PML protein is required for incorporation of the papillomavirus minor capsid protein L2 into virus-like particles. J. Virol. **78:** 1121–1128.
2. **Belnap, D. M., N. H. Olson, N. M. Cladel, W. W. Newcomb, J. C. Brown, J. W. Kreider, N. D. Christensen, and T. S. Baker.** 1996. Conserved features in papillomavirus and polyomavirus capsids. J. Mol. Biol. **259:**249–263.
3. **Bousarghin, L., A. Touze, P. Y. Sizaret, and P. Coursaget.** 2003. Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. J. Virol. **77:**3846–3850.
4. **Brady, J. N., V. D. Winston, and R. A. Consigli.** 1977. Dissociation of polyoma virus by the chelation of calcium ions found associated with purified virions. J. Virol. **23:**717–724.
5. **Chen, P. L., M. Wang, W. C. Ou, C. K. Lii, L. S. Chen, and D. Chang.** 2001. Disulfide bonds stabilize JC virus capsid-like structure by protecting calcium ions from chelation. FEBS Lett. **500:**109–113.
6. **Chroboczek, J., E. Gout, A. L. Favier, and R. Galinier.** 2003. Novel partner proteins of adenovirus penton. Curr. Top. Microbiol. Immunol. **272:**37–55.
7. **Chromy, L. R., J. M. Pipas, and R. L. Garcea.** 2003. Chaperone-mediated in vitro assembly of polyomavirus capsids. Proc. Natl. Acad. Sci. USA **100:** 10477–10482.
8. **Cripe, T. P., S. E. Delos, P. A. Estes, and R. L. Garcea.** 1995. In vivo and in vitro association of hsc70 with polyomavirus capsid proteins. J. Virol. **69:** 7807–7813.
9. **Day, P. M., D. R. Lowy, and J. T. Schiller.** 2003. Papillomaviruses infect cells via a clathrin-dependent pathway. Virology **307:**1–11.
10. **Drobni, P., N. Mistry, N. McMillan, and M. Evander.** 2003. Carboxy-fluorescein diacetate, succinimidyl ester labeled papillomavirus virus-like particles fluoresce after internalization and interact with heparan sulfate for binding and entry. Virology **310:**163–172.
11. **Ellgaard, L., and L. W. Ruddock.** 2005. The human protein disulphide isomerase family: substrate interactions and functional properties. EMBO Rep. **6:**28–32.
12. **Garcea, R. L., and P. A. Estes.** 1998. Purification of papovavirus virus-like particles from Sf9 insect cells, p. 521–527. In J. E. Celis (ed.), Cell biology: a laboratory handbook, 2nd ed., vol. 1. Academic Press, New York, N.Y.
13. **Garcea, R. L., D. M. Salunke, and D. L. Caspar.** 1987. Site-directed mutation affecting polyomavirus capsid self-assembly in vitro. Nature **329:**86–87.
14. **Ishizu, K. I., H. Watanabe, S. I. Han, S. N. Kanesashi, M. Hoque, H. Yajima, K. Kataoka, and H. Handa.** 2001. Roles of disulfide linkage and calcium ion-mediated interactions in assembly and disassembly of virus-like particles composed of simian virus 40 VP1 capsid protein. J. Virol. **75:**61–72.
15. **Li, M., P. Beard, P. A. Estes, M. K. Lyon, and R. L. Garcea.** 1998. Intercapsomeric disulfide bonds in papillomavirus assembly and disassembly. J. Virol. **72:**2160–2167.
16. **Li, M., T. P. Cripe, P. A. Estes, M. K. Lyon, R. C. Rose, and R. L. Garcea.** 1997. Expression of the human papillomavirus type 11 L1 capsid protein in *Escherichia coli*: characterization of protein domains involved in DNA binding and capsid assembly. J. Virol. **71:**2988–2995.
17. **Li, P. P., A. Nakanishi, S. W. Clark, and H. Kasamatsu.** 2002. Formation of transitory intrachain and interchain disulfide bonds accompanies the folding and oligomerization of simian virus 40 Vp1 in the cytoplasm. Proc. Natl. Acad. Sci. USA **99:**1353–1358.
18. **Magnuson, B., E. K. Rainey, T. L. Benjamin, M. Baryshev, S. Mkrtchian, and B. Tsai.** 2005. ERp29 triggers a conformational change in polyomavirus to stimulate membrane binding. Mol. Cell **20:**289–300.
19. **Mannova, P., and J. Forstova.** 2003. Mouse polyomavirus utilizes recycling endosomes for a traffic pathway independent of COPI vesicle transport. J. Virol. **77:**1672–1681.
20. **Mayer, M. P.** 2005. Recruitment of hsp70 chaperones: a crucial part of viral survival strategies. Rev. Physiol. Biochem. Pharmacol. **153:**1–46.
21. **McCarthy, M. P., W. I. White, F. Palmer-Hill, S. Koenig, and J. A. Suzich.** 1998. Quantitative disassembly and reassembly of human papillomavirus type 11 viruslike particles in vitro. J. Virol. **72:**32–41.

22. **Modis, Y., B. L. Trus, and S. C. Harrison.** 2002. Atomic model of the papillomavirus capsid. EMBO J. **21:**4754–4762.
23. **Niewiarowska, J., J. C. D'Halluin, and M. T. Belin.** 1992. Adenovirus capsid proteins interact with HSP70 proteins after penetration in human or rodent cells. Exp. Cell Res. **201:**408–416.
24. **Noiva, R.** 1999. Protein disulfide isomerase: the multifunctional redox chaperone of the endoplasmic reticulum. Cell Dev. Biol. **10:**481–493.
25. **Norkin, L. C., H. A. Anderson, S. A. Wolfrom, and A. Oppenheim.** 2002. Caveolar endocytosis of simian virus 40 is followed by brefeldin A-sensitive transport to the endoplasmic reticulum, where the virus disassembles. J. Virol. **76:**5156–5166.
26. **Pelkmans, L., T. Burli, M. Zerial, and A. Helenius.** 2004. Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. Cell **118:**767–780.
27. **Richterova, Z., D. Liebl, M. Horak, Z. Palkova, J. Stokrova, P. Hozak, J. Korb, and J. Forstova.** 2001. Caveolae are involved in the trafficking of mouse polyomavirus virions and artificial VP1 pseudocapsids toward cell nuclei. J. Virol. **75:**10880–10891.
28. **Saphire, A. C., T. Guan, E. C. Schirmer, G. R. Nemerow, and L. Gerace.** 2000. Nuclear import of adenovirus DNA in vitro involves the nuclear protein import pathway and hsc70. J. Biol. Chem. **275:**4298–4304.
29. **Sapp, M., C. Volpers, M. Müller, and R. E. Streeck.** 1995. Organization of the major and minor capsid proteins in human papillomavirus type 33 virus-like particles. J. Gen. Virol. **76:**2407–2412.
30. **Schaffhausen, B. S., and T. L. Benjamin.** 1976. Deficiency in histone acetylation in nontransforming host range mutants of polyoma virus. Proc. Natl. Acad. Sci. USA **73:**1092–1096.
31. **Selinka, H. C., T. Giroglou, and M. Sapp.** 2002. Analysis of the infectious entry pathway of human papillomavirus type 33 pseudovirions. Virology **299:**279–287.
32. **Shafti-Keramat, S., A. Handisurya, E. Kriehuber, G. Meneguzzi, K. Slupetzky, and R. Kirnbauer.** 2003. Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. J. Virol. **77:**13125–13135.
33. **Talcott, B., and M. S. Moore.** 1999. Getting across the nuclear pore complex. Trends Cell Biol. **9:**312–318.
34. **Tsai, B., C. Rodighiero, W. I. Lencer, and T. A. Rapoport.** 2001. Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. Cell **104:**937–948.
35. **Young, J. C., J. M. Barral, and F. Ulrich Hartl.** 2003. More than folding: localized functions of cytosolic chaperones. Trends Biochem. Sci. **28:**541–547.