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Active Foamy Virus Proteinase Is Essential for Virus Infectivity but Not for Formation of a Pol Polyprotein

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To analyze proteolytic processing of foamy (spuma) retroviruses, two mutations were generated in the presumed active-site triplet Asp-Ser-Gly in the predicted proteinase (PR) region of the human foamy virus (HSRV). The mutations changed either the presumed catalytic aspartic acid residue to a catalytically incompetent alanine or the adjacent serine to a threonine found in most cellular and retroviral proteases at this position. Both mutations were cloned into the full-length infectious HSRV DNA clone. Wild-type and S/T mutant genomes directed the synthesis of particles with similar infectious titers, while the HSRV D/A PR mutant was noninfectious. Immunoblot analysis of transfected cells revealed identical patterns for the wild-type and for the S/T PR mutant. HSRV D/A mutant-transfected cells expressed only a single Gag polyprotein of 78 kDa instead of the 78-kDa–74-kDa doublet found in HSRV-infected or wild-type-transfected cells. Analysis with *pol*-specific antisera yielded a protein of approximately 120 kDa reactive with antisera against *pol*- but not *gag*-specific domains. No Gag-Pol polyprotein was detected in this study. Electron microscopy analysis of transfected cells showed heterogeneous particle morphology in the case of the D/A mutant, with particles of normal appearance and particles of aberrant size and shape. These results indicate that foamy viruses have an aspartic PR that is essential for infectivity but not for formation of the 120-kDa Pol polyprotein.

Proteolytic processing of the structural Gag and Gag-Pol polyproteins by the virus-encoded virion-associated proteinase (PR) is essential for the production of infectious retroviral particles. Inactivation of PR, either by mutation of its active-site residues or by use of specific inhibitors, abolished retroviral infectivity and led to the release of immature-appearing noninfectious particles consisting of uncleaved viral polyproteins (12, 28). In the case of human immunodeficiency virus (HIV) and other retroviruses, the viral PR has been shown to cleave the Gag and Gag-Pol polyproteins at defined sites to produce all mature viral proteins (reviewed in references 13 and 15). Cellular proteases are believed not to be involved in retrovirus Gag and Gag-Pol polyprotein processing. Retroviral PRs belong to the family of aspartic proteases and are active as homodimers, with the active-site triplets (Asp-Thr/Ser-Gly; D-T/S-G) from both chains contributing to the symmetric active site of the enzyme (26). The catalytic apparatus of aspartic proteases consists primarily of the two aspartic acid residues within the conserved D-T/S-G triplets which are located in the center of the substrate-binding cleft and are essentially in the same spatial relationship in the three-dimensional structures determined to date (33; reviewed in reference 32). A particular feature of the active site is the side-chain-to-backbone interaction between the hydroxyl group of the Thr residue in the DTG triplet and the main chain of the other PR molecule in the dimer. These interactions form a network of two symmetrical pairs of hydrogen bonds, stabilizing the structure of the active site which has been described as "fireman's grip" (6). The only other small amino acid capable of forming such an interaction is serine, and a DSG triplet has been found in the PR region of avian retroviruses, yeast retrotransposons, and foamy viruses (30).

Foamy viruses (spumaviruses) are a group of complex retroviruses that have been isolated from a number of animal species and are believed to be apathogenic in their animal host (19). The human foamy virus (HSRV) was originally isolated from a patient with nasopharyngeal carcinoma (1). The HSRV proviral genome has been cloned and sequenced, and the genetic organization and some unusual aspects of its replication cycle have been studied (reviewed in references 16 and 20). Foamy virus morphogenesis involves the assembly of intracytoplasmic core particles, similar to B- and D-type retroviruses, which bud at the plasma membrane but preferentially at intracellular membranes (7). A particular feature of foamy virus replication is the fact that viral structural antigens remain mainly cell associated and few extracellular virions are found. Most intracellular particles appear to be immature, and analysis of foamy virus Gag proteins has revealed mostly precursor polyproteins, with the main Gag-reactive proteins being a doublet of 78 kDa-74 kDa and very limited amounts of processed products (3, 23). Furthermore, the sizes of *pol*-derived products corresponding to the viral PR, reverse transcriptase (RT)-RNase H, and integrase (IN) have been reported to be 10, 80, and 39 kDa, respectively (11, 24, 25). However, foamy virus polyprotein processing has not been studied in detail, and no analysis of the viral PR activity has been reported.

The amino acid sequence derived from the 5′ part of the foamy virus *pol* gene, predicted to be expressed by a +1 frameshift event unusual in retroviruses, contains a homologous DSG sequence that determines the location of the putative PR region (30). Outside the conserved active-site region (with a Ser residue instead of the more frequent Thr residue), there is a second sequence motif, GlyArgLys, corresponding to the pattern GlyArg(Asp/Asn) found in most other viral aspartic PRs (26). Apart from these characteristic features, foamy virus

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TABLE 1. Analysis of infectious titers of wt and mutated viruses[a]

Virus derived from proviral plasmid	Virus titers from expt no.:		
	1	2	3
pHSRV13	5×10^3	2×10^4	2×10^4
pHSRV-S/T	3×10^3	1×10^4	3×10^4
pHSRV-D/A	0	0	0

[a] BHK21 cells (experiments 1 and 3) or BHK FAB cells (which contain an integrated copy of the *lacZ* gene under the control of the HSRV long terminal repeat promoter [34]; experiment 2) were electrotransfected with 10 μg of DNA of the respective proviral clones as described previously (21). To normalize for transfection efficiency, 1 μg of an expression vector containing the *lacZ* gene under the control of the human cytomegalovirus promoter-enhancer (in the case of BHK21 cells) or 1 μg of a similar expression vector containing the luciferase gene (in the case of BHK FAB cells) was cotransfected (17). Cells and culture media were harvested 2 to 3 days after transfection. Culture media were cleared by low-speed centrifugation and diluted with fresh media to normalize for differences in transfection efficiencies. Serial dilutions of cleared media were added to fresh BHK FAB cells (20 to 40% confluent), and virus titer was determined by counting blue cells after in situ X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining 48 h after infection (34). For analysis of transfection efficiencies, transfected cells were lyzed in 25 mM Tris (pH 7.5)–4 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]–10% glycerol–1% Triton X-100, and postnuclear supernatants were used for determination of β-galactosidase activity (BHK21 cells) or luciferase activity (BHK FAB cells [17, 34]). Data from three independent transfections and titrations are shown for each proviral clone.

PR exhibits very little similarity to other retroviral and cellular proteases. In particular, a conserved structurally flexible region ("flap") that is normally found between the described conserved elements and that is involved in substrate binding cannot be easily identified in the foamy virus PR region. Considering the limited sequence similarity and the observation that foamy virus Gag proteins are mostly detectable as precursor proteins, we hypothesized that there may be special features in foamy virus polyprotein processing. In order to prove that foamy viruses contain an aspartic PR essential for virus replication and to analyze polyprotein processing, we generated a mutation which changed the presumed active-site Asp residue to an Ala residue and analyzed the phenotype of the resulting proviral clone pHSRV-D/A regarding virus infectivity, polyprotein processing, and particle morphology. A second mutation was based on the hypothesis that a PR containing a DTG instead of the DSG triplet may be more active and changed the Ser residue to the more commonly found Thr residue (pHSRV-S/T). The corresponding reverse mutation in the HIV type 1 (HIV-1) PR had been shown to reduce PR activity by a factor of 5 to 10 (12a).

The two mutations were generated by oligonucleotide-directed mutagenesis on a single-stranded DNA template according to the method of Kunkel (14). As the template, we used single-stranded DNA derived from plasmid pBS-H(N/P) that contains a *Nar*I-*Pst*I fragment of the infectious proviral plasmid pHSRV13 (nucleotides [nt] 1126 to 5063 of the HSRV genome) in pBluescript (Stratagene). The sequences of the two oligonucleotides used were as follows (altered nucleotides are underlined): 5′-CC CAC TGG GCT TCA GGG GC-3′ (D/A; nt 3178 to 3196 of the HSRV genome) and 5′-CAC TGG GAT ACA GGG GCA AC-3′ (S/T; nt 3180 to 3199 of the HSRV genome). Following mutagenesis, the *Dra*III-to-*Bsu*36I fragments (nt 3098 to 3631 of the HSRV genome spanning the putative PR region) of the D/A and S/T mutant plasmids were cloned into pHSRV13 (18) to yield plasmids pHSRV-D/A and pHSRV-S/T, respectively. The presence of the mutations was confirmed by sequence analysis of the region between the *Dra*III and *Bsu*36I sites.

To analyze the influence of the mutations in the putative PR active site on viral infectivity, we titrated the infectivity of virus particles harvested after transient transfection of BHK21 or BHK FAB cells (34) with plasmids pHSRV13, pHSRV-D/A, and pHSRV-S/T, respectively (Table 1). Cleared culture media collected 2 to 3 days after transfection were normalized for transfection efficiency, and serial dilutions were used to infect fresh HSRV-permissive BHK FAB cells. No infectious virus particles were recovered in four independent transfections with pHSRV-D/A proviral DNA, although similar transfection efficiencies were achieved, and synthesis of foamy virus proteins and particle assembly was easily detected in mutant-transfected cells (see below). Culture media from pHSRV-S/T-transfected cells, on the other hand, yielded titers similar to those obtained for the media from the wild-type (wt)-transfected cells (Table 1). On the basis of these results, we conclude that HSRV contains an aspartic PR essential for virus infectivity as observed in a number of other retroviral systems. Changing the active-site triplet to the more commonly found DTG triplet did not alter infectious titers, suggesting that the unusual Ser residue in the foamy virus PR active site does not significantly impair virus infectivity. Similarly, mutation of the PR active-site DSG of the yeast transposon Ty3 to the commonly found DTG had little if any effect on polyprotein processing and viral replication (10). Furthermore, a reverse mutation changing the HIV PR active site from DTG to DSG has been shown to reduce PR activity in in vitro assay systems by a factor of 5 to 10, with little effect on processing of particle-associated polyproteins and no change in virus infectivity (12a). Since this mutation primarily affected the K_m values, it was concluded that the observed reduced activity of the mutated PR may not be phenotypically relevant for proteolysis inside the virion, where substrate concentrations should be saturating.

Figure 1 shows an immunoblot analysis of extracts from

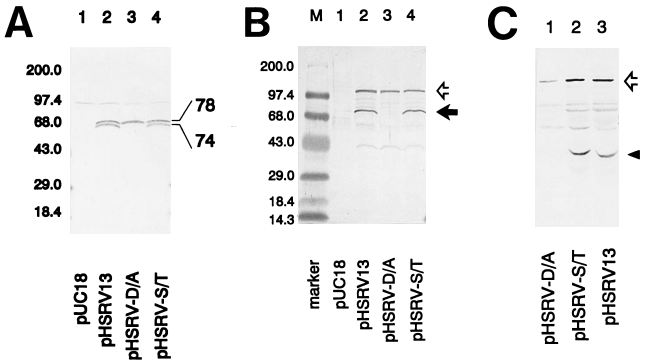

FIG. 1. Western blot analysis of transiently transfected BHK FAB cells with HSRV-specific antisera. Cells were lysed as described in Table 1 at 2 days after transfection with the proviral clones indicated. Protein samples normalized according to coexpressed β-galactosidase activity were analyzed on sodium dodecyl sulfate-polyacrylamide gels and electroblotted onto a nitrocellulose membrane (Schleicher & Schuel). Blots were reacted with polyclonal rabbit antisera against HSRV MA (A [5]), RNase H (B [11]), or IN (C [25]) proteins and were detected by color development as described previously (18). For the different blots, prestained marker proteins (BRL) shown in panel B with the molecular masses indicated in kilodaltons were separated in parallel. (A) The positions of the Gag precursor proteins of 74 and 78 kDa detected with the MA antiserum are indicated for both proteins. The mature MA protein is not visible in this blot because of poor proteolytic processing. (B and C) The blots were intentionally overdeveloped to demonstrate the absence of the mature 80-kDa RT-RNase H protein (B [solid arrow]) and the 39-kDa IN protein (C [arrowhead]) in extracts from pHSRV-D/A-transfected cells. The Pol polyprotein of about 120 kDa (B and C [open arrows]) was detectable in lysates derived from the wt- and mutant provirus-transfected cells.

BHK FAB cells transfected with pHSRV13, pHSRV-D/A, and pHSRV-S/T proviral clones, respectively. The blots were probed with antiserum against the N-terminal matrix (MA) domain of the Gag polyprotein (Fig. 1A) or against the *pol*-encoded RNase H (Fig. 1B) and IN (Fig. 1C) domains (5, 11, 25). Antiserum against MA predominantly detected two bands corresponding to the reported 78-kDa–74-kDa doublet of the Gag polyprotein (3, 23) in wt- and pHSRV-S/T-transfected cells but not in untransfected cells (Fig. 1A, lanes 2, 4, and 1, respectively). In contrast, only a single band comigrating with the upper (78-kDa) band of the doublet was observed in pHSRV-D/A-transfected cells (Fig. 1A, lane 3), suggesting that the lower band in the doublet is the result of proteolytic processing mediated by the viral PR. It is likely that the 78-kDa protein corresponds to the full-length Gag polyprotein (predicted length, 649 amino acids) which is cleaved by PR in the amino- or carboxy-terminal part to yield the 74-kDa species. Interestingly, in wt-transfected as well as in HSRV-infected cells, there is usually an approximately 1:1 ratio of the two proteins, independent of the time point analyzed. This processing step may, therefore, not normally go to completion, suggesting that there may be a need for the formation of 78-kDa–74-kDa heterodimers.

Panels B and C of Fig. 1 show immunoblot analyses of cellular extracts reacted with antisera against HSRV RNase H and IN, respectively. The RNase H domain is part of a single polypeptide chain together with RT, since RT- and RNase H-specific antisera reacted with a single protein species of 80 kDa in HSRV-infected cells (11, 24) and RT and RNase H activities copurified from simian foamy virus type 1 particles as a single 81-kDa protein (4). Such an organization of the *pol* gene is analogous to that of murine leukemia virus, in which both RT and RNase H activities reside in a single 80-kDa protein (29, 31). HSRV IN corresponds to the C-terminal domain of the *pol* region and was detected as a 39-kDa protein in infected cells or purified virions (24, 25). In good agreement with these results, proteins of approximately 80 kDa (Fig. 1B, lane 2) and approximately 39 kDa (Fig. 1C, lane 3) were detected in pHSRV13-transfected cells with antisera against RNase H and IN, respectively. Similar amounts of the same products were also observed in pHSRV-S/T-transfected cells (Fig. 1B, lane 4, and Fig. 1C, lane 2), indicating that there was no significant alteration in polyprotein processing. Besides these two gene products, both antisera also detected a presumed precursor protein of approximately 120 kDa (Fig. 1B, lanes 2 and 4; Fig. 1C, lanes 2 and 3) that was not detected by Gag-specific antisera (Fig. 1A and data not shown). This protein has been observed previously in HSRV-infected cells and is likely to correspond to a complete Pol polyprotein including the PR, RT-RNase H, and IN domains (predicted size, 1,151 amino acids). The 120-kDa Pol polyprotein was also detected in pHSRV-D/A-transfected cells with antisera against RNase H (Fig. 1B, lane 3) or IN (Fig. 1C, lane 1). However, the D/A mutant provirus did not give rise to the 80- and 39-kDa proteins corresponding to RT-RNase H and IN, indicating that at least these HSRV proteins are derived from proteolytic cleavage of the Pol polyprotein by the viral PR.

Unexpectedly, none of our antisera detected a larger polyprotein in pHSRV-D/A-transfected cells which might correspond to a Gag-Pol polyprotein (corresponding to an approximate mass of 200 kDa). Similar results were obtained upon radioimmunoprecipitation of metabolically labelled transfected cells: a large polyprotein of approximately 200 kDa could not be detected (data not shown). It is therefore unlikely that the lack of a large polyprotein in immunoblot analysis was simply due to poorer transfer efficiency of larger proteins. Moreover, in parallel experiments the HIV pr160$^{Gag\text{-}Pol}$ polyprotein was easily detected in cells transfected with a PR-defective HIV proviral clone and no intermediate *pol*-reactive species were observed in this case (12a). The absence of a detectable Gag-Pol polyprotein in pHSRV-D/A-transfected cells may suggest, but certainly does not prove, that such a polyprotein does not exist in foamy viruses. However, the detection of a 120-kDa *pol*-reactive species lacking Gag epitopes in cells expressing a PR-defective mutant indicates that the viral PR is not required to cleave such a polyprotein, if it is made. A PR-independent production of the Pol domain without Gag has not been reported previously for any retroviral system (9). The production of the 120-kDa Pol polyprotein can be explained in the following ways: (i) the putative Gag-Pol polyprotein is rapidly cleaved by a cellular enzyme not requiring viral PR or (ii) there is no Gag-Pol precursor in foamy virus polyprotein synthesis, and the observed Pol protein may be translated independently of Gag. Such a phenotype could depend on production of a *pol*-specific spliced RNA (which has not been detected in a detailed PCR analysis of HSRV mRNAs [22]) or on internal initiation of ribosomes on the genomic RNA to form a bicistronic mRNA. Interestingly, a Met residue, which might be used for initiation of Pol protein synthesis, is found close to the 5′ end of the *pol* reading frame and is conserved in other sequenced foamy viruses.

Besides the apparent lack of a Gag-Pol polyprotein and PR-independent production of the separate Pol domain, our data indicate that there is a difference in the processing of HSRV Gag and Pol polyproteins. Cleavage of the Pol polyprotein appears to proceed more efficiently, yielding significant amounts of proteolytic products (Fig. 1B and C), while the only efficient cleavage of the Gag polyprotein appears to remove a small peptide to yield the 74-kDa protein. No substantial amounts of further cleavage products were found in transfected cells in these experiments, although *gag*-derived proteins of 27- and 32-kDa have been detected in another study (2).

To analyze the effect of the two PR active-site mutants on particle morphogenesis and morphology, we performed thin-section electron microscopy of COS 7 cells, transiently transfected with pHSRV13, pHSRV-S/T, and pHSRV-D/A proviral plasmids. Electron micrographs of wt-transfected cells revealed intracellular particles within membrane compartments which appeared mostly spherical, indicating that they may correspond to immature virions (Fig. 2a and b). Similar results had been obtained for foamy virus-infected or pHSRV13-transfected cells (7, 18). Particles observed in pHSRV-S/T-transfected cells revealed a morphology similar to that of the wt particles. These particles were spherical and localized within intracellular membrane compartments (Fig. 2c) with rare budding structures at the plasma membrane (Fig. 2e). Budding structures showed a concentration of surface spikes, presumed to correspond to the viral envelope glycoproteins (Fig. 2e). Intracellular particles contained frequently smooth membrane surfaces (Fig. 2d), but surface projections were also observed occasionally on intracellular particles (e.g., Fig. 2f). The particles found in wt- and S/T mutant-transfected cells appeared homogeneous regarding the size and morphology of the spherical core structure, with minor size differences probably caused by the plane of sectioning. Cells transfected with the pHSRV-D/A proviral clone, on the other hand, yielded intracellular particles of considerable heterogeneity in size and shape (Fig. 2g and h). These particles were also localized within membrane vesicles. While some particles closely resembled wt HSRV (Fig. 2h and k), there were also considerably larger particles with up to a twofold increase in particle diam-

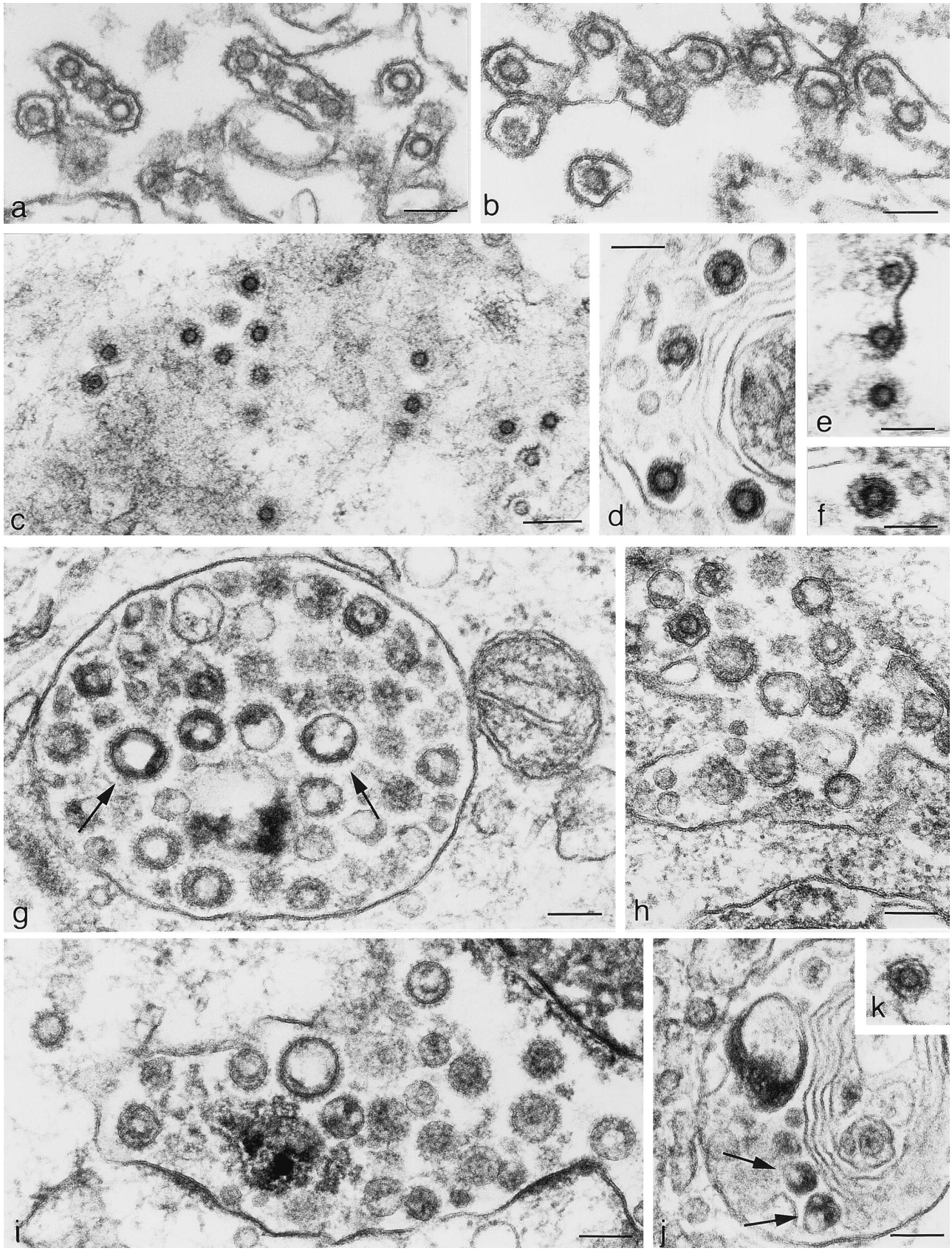

FIG. 2. Electron micrographs showing thin sections of COS 7 cells transfected with pHSRV13 (a and b), pHSRV-S/T (c to f), or pHSRV-D/A (g to k). At 48 h after transfection, the cells were fixed in situ with 2.5% glutaraldehyde in 50 mM sodium cacodylate (pH 7.2) for 30 min at 4°C. Fixed cells were scraped from the plate, collected by low-speed centrifugation, successively stained with 2% osmium tetroxide–0.5% uranyl acetate, and processed for ultrathin sectioning. Micrographs were taken with a Zeiss EM-10 electron microscope at 80 kV. Magnification, ×54,000 (panel c); ×90,000 (all other panels). Bar, 200 nm (panel c) and 100 nm (all other panels).

eter (Fig. 2g [arrows]). Furthermore, aberrant particle morphologies with open shell structures were frequently observed (Fig. 2j [arrows]). These aberrant particles are likely to be caused by a late arrest in morphogenesis, which prevents the complete closure of the spherical core particle and leads to the release of enveloped but incompletely closed particles. Similar morphologies have been reported for PR-defective mutants of HIV-1 (21, 27) and for PR-inhibitor-treated HIV-1 particles (8). These observations suggest that PR activity may not only be required for condensation of the viral core during virion maturation but may also play a role in an earlier step of virus morphogenesis leading to the formation of the spherical immature core particle.

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