The Carboxy-Terminal \( p3^{\text{Gag}} \) Domain of the Human Foamy Virus Gag Precursor Is Required for Efficient Virus Infectivity

Motomi Zemba, Thomas Wilk,*, Twan Rutten,*, Andrea Wagner, Rolf M. Flügel, and Martin Löchelt1

Abteilung Retroviral Geneexpression, Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, and *Structural Biology Programme, European Molecular Biology Laboratory, Heidelberg, Germany

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Proteolytic processing of foamy virus Gag proteins appears to be different from that of other retroviruses. A single carboxy-terminal cleavage site that is consistently detectable in human foamy virus (HFV) Gag precursor protein \( p74^{\text{Gag}} \) derived from infected cells and/or purified virus particles. Using a recombinant HFV protease, we have determined the carboxy-terminal p\( 3^{\text{Gag}} \) cleavage site that results in \( p70^{\text{Gag}} \) and the carboxy-terminal p\( 3^{\text{Gag}} \) (Pfrepper et al., 1997, Biochem. Biophys. Res. Commun. 237, 548–553). To study the biological functions of p\( 3^{\text{Gag}} \), proviral DNA clones were constructed coding for a carboxy-terminally truncated \( p70^{\text{Gag}} \) lacking the entire p\( 3^{\text{Gag}} \) protein. Removal of p\( 3^{\text{Gag}} \) resulted in an about 100-fold lower virus titer. The expression of other HFV proteins and the processing of Pol proteins were indistinguishable from those of wild-type-transfected cells. The defect in viral infectivity of the p\( 3^{\text{Gag}} \) mutants was partially restored by coexpressing the full-length p\( 74^{\text{Gag}} \) protein in trans. The deletion of p\( 3^{\text{Gag}} \) resulted in particle assembly with wild-type virion morphology and encapsidation of Pol proteins. Our data show that the carboxy-terminal p\( 3^{\text{Gag}} \) protein has an important function for viral infectivity but is not required for preassembly of capsids, virus morphogenesis, and incorporation of Pol proteins into virions.

1 To whom correspondence and reprint requests should be addressed at Abteilung Retrovirale Geneexpression, Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D - 69009 Heidelberg, Germany. Fax: 49-6221-42-4865. E-mail: m.loechelt@dkfz-heidelberg.de.

INTRODUCTION

Foamy viruses (FV) or spumaretroviruses constitute a separate group within the family of Retroviridae. The replication strategy of FVs has distinct aspects that are different from that of other retroviruses (Coffin, 1996; Rethwilm, 1995; Yu et al., 1996). FVs do not express their Pro-Pol proteins (protease, PR; reverse transcriptase, RT; RNase H, and integrase, IN) as part of a Gag-Pol precursor but use instead a spliced transcript to express the enzymatic functions independent of Gag (Bodem et al., 1996; Enssle et al., 1996; Jordan et al., 1996; Löchelt and Flügel, 1996; Yu et al., 1996). It has been postulated that, as a consequence of the unique mechanism of Pro-Pol expression, proteolytic processing of the \( p74^{\text{Gag}} \) precursor is inefficient, temporally delayed, or a combination of both (Giron et al., 1997; Winkler et al., 1997; Yu et al., 1996). The HFV PR-mediated cleavage of \( p74^{\text{Gag}} \) that is consistently detectable in cell-associated and/or cell-released Gag antigen is close to the carboxy terminus of Gag; additional Gag cleavage products have also been described (Bartholomaus et al., 1992; Enssle et al., 1997; Hahn et al., 1994; Komvalinka et al., 1995; Löchelt and Flügel, 1996; Morozov et al., 1997; Netzer et al., 1990). Using a recombinant HFV PR, we have experimentally determined the carboxy-terminal Gag cleavage site to be SerArgAlaValAsn → ThrValThrGln (the vertical arrow represents the cleavage site between the peptide bonds) consistent with predictions based on protein sequence alignments of known FV Gag sequences (Pfrepper et al., 1997; Winkler et al., 1997). In a recent study, mutations were introduced directly at, the authentic cleavage site (Enssle et al., 1997, Fischer et al., 1998). Here we describe the phenotype of HFV proviral clones that express a Gag protein specifically shortened at the cleavage site. We show that the p\( 3^{\text{Gag}} \) protein is essential for viral infectivity butispensable for particle morphogenesis, morphology, and packaging of Pol proteins into virions.

RESULTS

Removal of the p\( 3^{\text{Gag}} \) protein domain strongly reduces HFV infectivity

To determine the biological role of the carboxy-terminal p\( 3^{\text{Gag}} \) protein, this domain was deleted from HFV gag by introducing two stop codons immediately downstream of the p\( 3^{\text{Gag}} \) cleavage site by site-directed mutagenesis. Two independent clones designated pHSRVΔp4

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(subclones 1 and 5) were shown to contain the mutations introduced and were used throughout this study.

Two days after transfection of 10 μg of wt and each p3-deleted proviral clone into permissive BHK-21 cells, strong syncytia formation was observed independently of whether the terminus of Gag had been deleted. After low-speed centrifugation, cell culture supernatants were used to determine viral infectivity on FAB cells (Yu and Linial, 1993). Whereas wt pHSRV13-transfected cells yielded a virus titer of about 1 × 10^5, the infectivity of both p3 deletion mutants was repeatedly found to be 50- to 100-fold lower. This result shows that the proteolytic cleavage at the p3 site, p3Gag itself, or the full-length p74Gag precursor has an important role for infectivity. Previous studies showed that mutants having nonconservative amino acid changes close to the p3 processing site were not infectious at all (Enssle et al., 1997).

Transcomplementation of full infectivity of mutants

In order to transcomplement full infectivity of mutants pHSRVΔp4, the full-length authentic HFV p74Gag was coexpressed from the eukaryotic expression construct pBCgag in trans. Coexpression of 1 μg of either pHSRVΔp4 mutant together with 9 μg of a wt HFV p74Gag expression plasmid resulted in a clear increase in viral infectivity when compared to cotransfections with pUC18 control DNA (Table 1). However, cotransfections with the Gag expression clone did not result in restoration of full infectivity when compared to transfection of 1 μg of the wt provirus pHSRV13 DNA.

Removal of the p3Gag protein does not alter HFV gene expression

We next investigated whether the overall expression of HFV genes and the proteolytic processing of Pro-Pol were altered by deleting the terminal Gag sequences. For this purpose, cell-associated antigens were analyzed by immuno blotting of cell extracts harvested 48 h after transfection with pHSRVΔp4, pHSRV13, and pUC18 control DNAs. Aliquots of cells transfected with similar efficiency were analyzed by immunoblotting. When a CA/NC-specific antiserum was used, clone pHSRVΔp4 was found to express a truncated 70-kDa Gag protein (Fig. 1A, lane 3, thin arrow) that comigrated with the partially processed p70Gag protein from wt-transfected cells (lane 2). As expected, the p74Gag precursor (solid arrow) was not detectable in cells transfected with pHSRVΔp4, whereas it was clearly visible in extracts from wt-transfected cells. There were no

Table 1

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<th>2 days p.i.</th>
<th>3 days p.i.</th>
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<tr>
<td>pHSRV13 wt plus pUC18</td>
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<td>16,000</td>
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<tr>
<td>pHSRVΔp4 plus pUC18</td>
<td>88</td>
<td>50</td>
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<tr>
<td>pHSRVΔp4 plus pCMVGag</td>
<td>1875</td>
<td>530</td>
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* The HFV infectivity released from transfected cells was quantitated as described under Materials and Methods using FAB indicator cells.

** Samples for titrations were taken 2 and 3 days after transfection, as indicated.

FIG. 1. Deletion of the terminal Gag p3 protein does not influence HFV gene expression. Extracts from BHK-21 cells transfected with plasmids pUC18 (lanes 1), pHSRV13 (lanes 2), and pHSRVΔp4 (lanes 3) were harvested 48 h after transfection. Aliquots were subjected to immunoblotting using HFV CA-NC (A)-, RNase H (B)-, and Bel 1/Bet (C)-specific antisera. The positions of the 74-kDa unprocessed Gag precursor (solid arrow), the p70Gag (light arrow), the 127-kDa Pro-Pol precursor (arrowhead), B, the 80-kDa RT-RNase H (asterisk), and the 56-kDa Bet protein (diamond, C) are marked at the right margins. Apparent sizes of prestained protein molecular mass markers are shown in kDa in the left margins.
significant differences in the level of Gag expression when comparing wt and p3-deleted provirus transfections. Extracts from pUC-transfected cells did not contain HFV-specific proteins.

When using antisera directed against the corresponding HFV proteins, clones pHSRVΔp4 (lanes 3) and pHSRV13 (lanes 2) showed similar levels of protein expression and processing of Pro-Pol (Fig. 1B), Bel 1/Bet (Fig. 1C), and Env (data not shown). This indicates that the overall expression of HFV structural and bel genes was not altered and that processing of Pro-Pol and Env proteins was not changed by the deletion of the carboxy-terminal p3Gag protein.

Removal of the p3Gag protein does not affect virus morphology

We next studied the morphology of HFV particles in cells transfected with wt and p3-deleted HFV proviruses. Cells were grown for 2 days after transfection on coverslips before processing for thin-section electron microscopy.

Under the electron microscope, pHSRV13 wt-transfected cells revealed numerous small and large vacuoles (spongy appearance). Several classes of virus particles were identified. Within the dense cytoplasm and often within the immediate vicinity of the nuclear membrane (but not within the nucleus), large numbers of capsids with a diameter of 40±50 nm were present. They were sometimes organized into tightly packed clusters (not shown) which resided outside the ER. Another indication of these clusters being outside the ER was that the individual capsids were only a few nanometer apart and did not possess a membrane. Those virus particles that had entered the ER were organized into rows, sheets, or what seemed to be pairs (Fig. 2a). When clearly visible, the viral membrane often enveloped multiple capsids (see Fig. 2b). Within the ER, the viral membrane often appeared smooth (Fig. 2a). However, the existence of a clear lumen between viral and ER membrane (see Fig. 2b) seems indicative of the presence of a spiky coat. A prominent, well-stained, spiky coat was observed in those virus particles located in vacuoles of the spongy cytoplasm (Fig. 2c) and especially when they were shed into large cellular vacuoles (Fig. 2d) or into the medium (not shown). Occasionally, however, a coat with a complete set of spikes was absent (Figs. 2d and 2e). Interestingly, even within the large vacuoles, virus particles often occurred in pairs (Fig. 2d).

Similar to the control, cells transfected with the pHSRVΔp4 mutant showed the formation of spongy cytoplasm. Higher magnifications revealed virus particles which were indistinguishable from the wt (Fig. 3a). As in the control, clusters of capsids apparently lacking a membrane were found within dense cellular cytoplasm (Fig. 3b). Large individual virions with a distinct outer membrane bearing Env proteins appeared in the spongy cytoplasm but especially in the large intracellular vacuoles (Fig. 3a).

Removal of the p3Gag protein does not affect release and composition of HFV particles

The removal of the p3Gag protein might interfere with the release of viral particles and/or their composition. To investigate this aspect, supernatants of transfections with wt pHSRV13 DNA and transfections of the p3-deleted HFV provirus in the absence or the presence of coexpressed full-length HFV Gag were analyzed for HFV particles. Corresponding cell culture supernatants were harvested 2 and 3 days after transfection, cleared by low-speed centrifugation, layered onto a 20% sucrose cushion, and centrifuged for 2 h at 76,000 g at 4°C. The resulting virus pellets were re-suspended in protein sample buffer and subjected to immunoblotting using the antisera described above. To enhance the sensitivity of the detection system, the immunoblots were developed with an enhanced chemoluminescence system. Immunoblotting with the CA/NC-specific antiserum showed that slightly lower amounts of p70Gag were released from pHSRVΔp4-transfected cells (Fig. 4A, lanes 2 and 5) when compared to the amount of p74 and p70Gag from pHSRV13-transfected cells (Fig. 4A, lanes 1 and 4). As expected, the HFV p74Gag precursor was absent from pHSRVΔp4-transfected cells, whereas low amounts of full-length Gag were incorporated into viral particles when p74Gag was provided in trans by a cotransfected HFV full-length Gag expression plasmid (Fig. 4A, lanes 3 and 6). To analyze whether Pol and Env proteins are also detectable in released wt and mutant viruses, immunoblots were performed with RNase H (Fig. 4B)- and Env-specific (data not shown) antisera. RNase H-specific antigen corresponding to the 80-kDa PR-RT-RNase H molecule was clearly detectable in cells from p3-deleted transfections and wt transfections (Fig. 4B, lanes 1 to 4 marked by an asterisk). The unprocessed pre127Pro-Pol precursor was almost absent from purified virions, confirming that proteolytic processing of Pro-Pol occurred independently of whether p3 was present. The uncleaved pre127Pro-Pol (arrowhead, Fig. 4B) was clearly present in cell-associated viral antigen (lane at the left margin) but almost absent in virus preparations (lanes 1 to 4). This indicates that the one-step enrichment of virions was both efficient and effective. In addition, the presence and the processing of Env as part of released HFV particles was not significantly affected by the Gag mutation (data not shown). These results suggest that the p3Gag domain either in the Gag precursor or by itself is not involved in Pol processing and/or Pol and Env incorporation into the virion.
FIG. 2. Morphology of wt HFV 2 days after transfection of pHSRV13 DNA into BHK-21 cells. (a) Accumulation of virus particles, often within the vicinity of a nucleus (n); capsids which often occur in pairs (arrowheads) are surrounded by membranes (arrows) indicating that they reside within the ER. (b) Pair of capsids surrounded by a single viral membrane (large arrowhead) located within the lumen of the ER (small arrowheads indicate ER membrane). (c) Virus particles with a very distinct coat of spikes (arrowheads) within the vacuoles of the spongy cytoplasm. (d) Budding and shedding of virus particles into a large vacuole (v); again the particles seem to occur in pairs. (e) Budding of a virus into a vacuole of the spongy cytoplasm. The bar corresponds to 200 nm.

FIG. 3. Morphology of p3-deleted HFV 2 days after transfection of pHSRVΔp4 DNA into BHK-21 cells. (a) Budding and shedding of virus particles into a large vacuole (v); the outer membrane of most particles is decorated with prominent spikes (arrowheads); note the similarity to Fig. 2d; compare also the small tightly packed cluster of capsids in the lower center of the figure. (b) Clusters of tightly packed capsids apparently lacking a membrane within the dense cytoplasm of a transfected cell. The bar corresponds to 200 nm.
FIG. 4. Detection of HFV particles in cell culture supernatants of transfected cells by immunoblotting. (A) BHK-21 cells were transfected with pHSRV13 (lanes 1 and 4), pHSRVΔp4 (lanes 2 and 5), and pHSRVΔp4 plus an HFV Gag expression plasmid (lanes 3 and 6). Cell culture supernatants were harvested 2 days after transfection and virus particles were purified by sedimentation through a sucrose cushion. Identical aliquots of pelleted HFV particles were subjected to immunoblotting with an HFV CA/NC-specific antiserum. Cell-associated antigen from HFV- and mock-infected HEL299 cells were used as controls. The solid arrow indicates the position of the 74-kDa, the light arrow that of the p70Gag proteins. The positions of marker proteins are given by dots at the left margin of the blots: phosphorylase B, 111 kDa; bovine serum albumin, 74 kDa; ovalbumin, 45 kDa (from top to bottom). (B) BHK-21 cells were transfected with pHSRV13 (lane 1), pHSRVΔp4 (lane 2), and pHSRVΔp4 plus an HFV Gag expression plasmid (lanes 3 and 4). Cell culture supernatants were harvested 2 days after transfection and virus particles were purified as above. Identical aliquots of pelleted virus proteins were subjected to immunoblotting with an HFV RNase H-specific antiserum. Cell-associated antigens from HFV- and mock-infected HEL299 cells were used as controls. The arrowhead marks the position of the 127-kDa Pro-Pol precursor and the asterisk that of the 80-kDa Pro-RT-RNase H proteins.

DISCUSSION

In this study we investigated the biological role of the carboxy-terminal p3Gag protein for HFV gene expression, infectivity, and virus morphogenesis and morphology. Our data show that removal of the p3Gag moiety results in a strong decrease in virus titer. In contrast, HFV gene expression, proteolytic processing of the Pro-Pol precursor protein, preassembly of viral capsids, and overall virus morphology as well as virus release and incorporation of Pol and Env proteins were apparently not altered or these changes were not detectable by the methods employed. In contrast to previous investigations on HFV morphogenesis (Enssle et al., 1997; Fischer et al., 1998), the p70Gag protein in our experimental system was specifically terminated at its authentic cleavage site (Pfrepper et al., 1997). Intentionally, we did not use heterologous overexpression systems to study HFV morphology and morphogenesis as described previously (Enssle et al., 1997; Fischer et al., 1997, 1998).

Our data indicate, that the carboxy-terminal domain of the FV Gag precursor protein has a function either very late during assembly and/or that this domain is critically involved in functions required upon infection of new host cells. Since virus release and particle composition seemed unchanged, we favor the latter explanation. Removal of the p3Gag protein does not prevent reverse transcription of HFV proviral DNA species (data not shown), in line with a previous report (Enssle et al., 1997). It is tempting to speculate that information contained within the short p3Gag sequence is required for penetration, disassembly, or proper functioning and targeting of the preintegration complex. A recent study indicated that proteolytic processing of Gag proteins takes place upon infection of new host cells (Giron et al., 1997). It remains to be seen whether the p3 domain may play a critical role in this process. It is worth mentioning that released HFV particles consist of a mixture of the full-length p74Gag and the partially processed p70Gag proteins (Fischer et al., 1998). Apparently, both Gag molecules have to be present in virus particles for proper infectivity as supported by our trans-complementation studies (Table 1). HFV particles consisting only of the Gag precursor exhibited severe defects in virus morphology characterized by significant size heterogeneity tending to oversized and apparently labile particles (Konvalinka et al., 1995; Fischer et al., 1998). This may be related to the fact that particles consisting exclusively of p74Gag do not achieve a wt curvature of the interacting Gag molecules to form a closed spherical capsid shell with a defined size of about 50 nm. The incorrect interactions of p74Gag molecules in these mutant particles may allow only a lower inner curvature of the forming capsid, causing the aberrant morphology observed as discussed for HIV (Fuller et al., 1997). In contrast, our present study shows that particles consisting exclusively of the authentic p70Gag molecules preassemble and bud indistinguishably from wt particles but have a severely reduced infectivity. The loss of infectivity (Table 1) may be related to the fact that the p70Gag particles have the correct size but are too stable for efficient disassembly processes. Thus, a balanced ratio of p70 and p74 Gag proteins or at least a minimal amount of the full-length p74Gag may be required for both proper assembly and disassembly (Table 1).

In another study, a heterologously overexpressed p70Gag protein, terminated a few amino acid residues upstream of the authentic p3 cleavage site, showed also an apparently unaltered particle formation (Fischer et al., 1998).

The function of the FV carboxy-terminal Gag domain is probably distinct from the late (L) function of other retroviruses, since HFV particles devoid of p3Gag do not have defects in release from cells as typical for L-domain mutants of other retroviruses (reviewed by Craven and Parent, 1996). Since p3-deleted particles exhibit wt levels of Pol and Env proteins, the terminus of p74Gag is not
required for packaging of Pro-Pol or incorporation of Env proteins into the viral membrane and is also not a determinant for the particle size, as recently identified for Rous sarcoma virus (Krishna et al., 1998).

Further experiments will be required to determine the function of the p3\textsuperscript{Gag} that does not exhibit strong sequence conservation among the known FVs (Winkler et al., 1997).

**MATERIAL AND METHODS**

**Cells, virus, cell culture, and electroporation**

Methods to propagate BHK-21 cells, to transfect them with recombinant HFV proviral DNAs, and to determine the HFV titer using the FAB titration assay have been described previously (L"echelt et al., 1991; Yu and Linial, 1993).

**Construction of recombinant clones**

For construction of specifically mutated HFV DNA clones, standard methods in molecular biology were employed (Sambrook et al., 1989). To construct an eukaryotic HFV p70\textsuperscript{Gag} expression plasmid, HFV DNA sequences from nt 1223 to 3173 were amplified by PCR using sense primer H1208s 5’-GAAAGCTTAGCCACCATGGAATTTAGTCC-3’ and antisense primer H3182a 5’-GTGGGATCCTCAATTTAGTCC-3’, and Pfu polymerase as recommended by the supplier (Stratagene, Heidelberg, Germany). The reaction product of about 19 kbp length was digested with HindIII and BamHI and inserted into the correspondingly digested expression vector pBC12CMV-Il2 (Cullen, 1986). The identities of two independently obtained HFV Gag expression clones, pBCgag2 and -7, were verified by DNA sequencing.

To create an HFV DNA clone that has the capacity to express the partially processed p70\textsuperscript{Gag} protein, two stop codons were introduced by PCR into gag directly downstream of the carboxy-terminal Gag cleavage site. For this purpose, PCR reactions using primers 2775s (L"echelt and Fl"ugel, 1996) plus dp4a 5’-TGATAGACACGCGTGAGTC-3’ and dp4s 5’-ACACAGAGTGCCACGTC-3’ (introduced stop codons in boldface type) plus primer 3486a and pHSVr13 DNA as template were performed as above (L"echelt and Fl"ugel, 1996). The gel-purified reaction products were combined and a third PCR with primers 2775s and 3486a was performed. The reaction product of about 700 nt contains two stop codons directly downstream of the carboxy-terminal PR cleavage site in Gag (boldface letters in primer pd4s) and was digested with SmaI and SalI and inserted into the corresponding restriction enzyme sites of pHHSVr13 by a three component ligation. Recombinant clones were analyzed by restriction analysis and DNA sequencing and were found to correspond to the wild-type (wt) nt sequence except for the mutations introduced.

Preparation and analyses of cell-associated and released HFV antigen

Cell-associated HFV antigen was prepared and analyzed by immunoblotting as described previously (L"echelt et al., 1991; L"echelt and Fl"ugel, 1996). Released viral particles were extracted from precleared cell culture supernatants by sedimentation through a 20% sucrose cushion in 100 mM NaCl, 10 mM Tris/HC, pH 7.4, 1 mM EDTA (wt/v) of 5 ml in an SW28 rotor (Beckman, Munich, Germany) for 2 h at 24,000 rpm, 4°C. The sediment was resuspended in double-concentrated protein sample buffer and analyzed by immunoblotting. The monospecific polyclonal antisera directed against defined domains of HFV structural and nonstructural proteins and methods used for protein transfer and detection by chromogens and chemiluminescence have been described previously (L"echelt and Fl"ugel, 1996).

**Cytology by electron microscopy**

For conventional electron microscopy, cells grown on coverslips were processed 2 days after transfection with plasmid DNA. The cells were briefly rinsed with phosphate-buffered saline and then fixed with 1% glutaraldehyde and 4% formaldehyde in PIPES buffer (0.25 M PIPES, pH 7.0) for 2 h. After three 15-min washes with the same buffer, the cells were postfixed with 1% OsO\textsubscript{4} in PIPES buffer for 1 h. The cells were then washed again with buffer and dehydrated in a graded ethanol series followed by embedding in Spurr's low-viscosity resin. After thin sectioning, samples were stained with uranyl acetate and lead citrate. Micrographs were taken on a Philips EM400 electron microscope.

**ACKNOWLEDGMENTS**

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