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Expression and Maturation of Human Foamy Virus Gag Precursor Polypeptides

MARIE-LOUISE GIRON,* SANDRINE COLAS, JANINE WYBIER, FRANCIS ROZAIN, AND RODICA EMANOIL-RAVIER

UPR 9051, CNRS "Pathologie cellulaire: aspects moléculaires et viraux," Hopital Saint-Louis, 75475 Paris Cedex 10, France

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In this report, we address the processing of the Gag polypeptides of human foamy virus previously reported to be atypical. In the cytoplasm or the nucleus of infected cells as well as in free virus particles, two Gag precursor polypeptides were identified at approximately 72 and 68 kDa, p72 giving rise to p68 by a maturation process. Efficient maturation of Gag precursors was observed only in two situations: (i) during the early steps of virus adsorption and (ii) under experimental conditions, including treatment with DNase I, known to dissociate actin polymers associated with high ionic strength and ionic detergents. Rather than being a defective viral protease function, an association of Gag precursors with a cytoskeleton network might be responsible for the low rate of Gag protein maturation through inhibition of their cleavage by the protease.

Foamy viruses belong to a large subfamily of complex retroviruses which induce in tissue culture a characteristic foamlike cytopathic effect. These viruses have a complex genomic organization, like lentiviruses and human oncoviruses. Foamy viruses are present in various mammalian species, giving rise to persistent infections without apparent pathogenicity (11).

The human foamy virus prototype (HFV) was isolated from nasopharyngeal carcinoma cells (1). In addition to the *gag*, *pol*, and *env* genes, it contains a regulatory region, named *bel*, in the 3′ end of its genome which may theoretically encode six different products (24). Among them, only Bel1 has been functionally characterized and shown to transactivate both its HFV promoters (long terminal repeat and an internal promoter in the *env* gene) (14, 18, 30, 37) and the heterologous long terminal repeat of human immunodeficiency virus (HIV) (13, 17). The other Bel proteins identified are Bel2 and Bet, whose functions are presently unknown (8, 19).

We and others have observed mainly Gag precursors and almost never the presence of mature Gag proteins in HFV-infected cells (2, 3, 25). In contrast, proteolytic processing of Pol proteins by HFV protease seems to occur normally (16). In this paper, we focus on the analysis of HFV Gag protein processing and suggest that tight binding of Gag polypeptides to the cytoskeleton blocks access to the protease.

The biosynthesis of viral proteins was monitored in the human glioblastoma cell line U373-MG by immunoprecipitation with specific antisera, as previously described (8). Polyclonal antisera against whole virus were produced by immunization of rabbits with infected cell lysates. Anti-Gag antisera raised against recombinant proteins were kindly provided by A. Aguzzi (Zürich, Switzerland) (anti-MA and anti-CA/NC) and by N. Morin (Montpellier, France) (anti-MA).

When HFV-infected cells are immunoprecipitated with either MA or CA antibodies, a doublet at 68 and 72 kDa is specifically detected (Fig. 1A). These proteins can also be seen with a polyclonal anti-HFV serum and were previously proposed to correspond to uncleaved Gag precursors. A kinetic analysis of late-stage HFV-infected cells revealed that (i) p72 was the first protein synthesized (as determined after a 10-min labeling) and p68 appeared only at 3 h postlabeling (data not shown) and (ii) no cleaved Gag proteins were ever observed up to 16 h postlabeling. These observations suggest that p68 derives from p72 and that the Gag precursors are not cleaved in this experimental setting. To demonstrate this, a pulse-chase experiment was performed with the two Gag-specific antisera. As shown in Fig. 1B, p72 was the only Gag polypeptide identified by each of the two antisera in the cytoplasmic extracts prior to chase. In contrast, p68 was detected only after a 2-h chase. No mature core proteins were ever observed, as they are in standard immunoprecipitation. Thus, in the cytoplasm p68 is the main maturation product of p72, even though the cleavage site has not been determined. Using a protease-deficient virus, Konvalinka et al. have shown that p68 appearance is blocked, suggesting that the viral protease is responsible for the maturation from p72 to p68 (16).

As free viral particles also present uncleaved precursors (data not shown), we examined the behavior of HFV Gag proteins after they entered host cells. Virus stocks labeled with [^{35}S]methionine-[^{35}S]cysteine were prepared, and the fate of viral polypeptides was monitored postinfection. One hour postadsorption, no cleavage of Gag precursors was observed in the cytoplasm. However, from 2 h on, new HFV polypeptides were found at 56, 32, 26, and 18 kDa (Fig. 2); these were identified as a Gag intermediate (MA+CA), CA, MA, and NC, respectively, by specific antisera (data not shown). Note that NC is clearly visible here, possibly due to the double [^{35}S]methionine-[^{35}S]cysteine labeling (NC does not contain methionine). To our knowledge, this is the first demonstration of spontaneous Gag protein cleavage for HFV. That it should occur during the import of infecting virus is highly atypical. The basis for this postinfection processing is not known but may involve specific interactions with host cell components.

Next we looked for the presence of incoming viral proteins in the nuclei of these infected cells. Nuclei were prepared and lysed in the presence of detergents (1% Triton X-100, 0.5% deoxycholate [DOC], 0.05% sodium dodecyl sulfate [SDS]) and a high concentration of salt (0.66 M NaCl), accompanied by DNase I treatment, to optimize the recovery of nuclear proteins. Up to 70% of the incoming viral proteins were re-

* Corresponding author. Mailing address: UPR 9051, CNRS "Pathologie cellulaire: aspects moléculaires et viraux," Hopital Saint-Louis, 1, Ave. Claude Vellefaux, 75475 Paris Cedex 10, France. Phone: 33 1 42401382. Fax: 33 1 42064857.

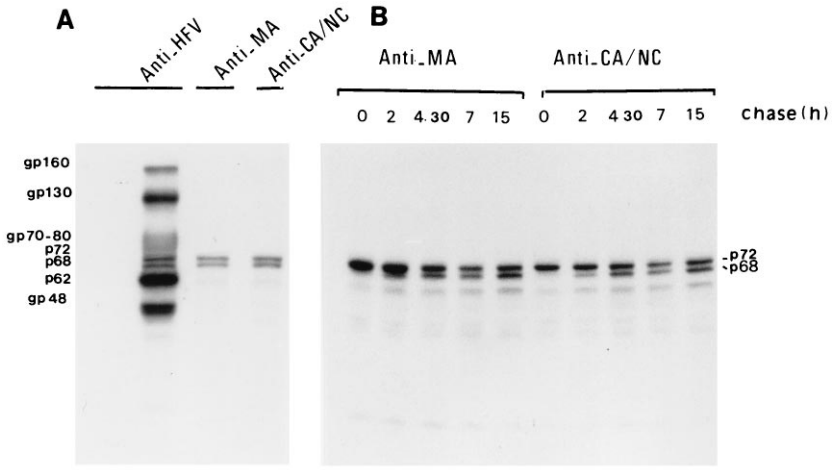

FIG. 1. Identification and processing of HFV Gag polypeptides. (A) HFV-infected cells, presenting an extensive cytopathic effect (6 to 8 days postinfection), were labeled with [^{35}S]methionine (50 μCi/ml; specific activity, 1200 Ci/ml; NEN) for 16 h in minimum essential medium without methionine but with 5% fetal calf serum. (B) For pulse-chase experiments, cells were labeled for 1 h with 170 μCi of [^{35}S]methionine per ml and placed in normal culture medium for 2, 4.5, 7 and 15 h. After being labeled, cells were lysed in a solution containing 0.05 M Tris (pH 7.4), 0.1 M NaCl, 0.005 M $MgCl_2$, 1% Triton X-100, 0.5% DOC, 0.05% SDS, and 0.003 M phenylmethyl sulfonyl fluoride (about 3×10^6 cells per 250 ml) for 30 min at 4°C. The supernatant recovered after centrifugation (5 min at 12,000 × *g*) was immunoprecipitated either with anti-whole-virus antiserum or specific anti-Gag antisera and resolved by SDS-polyacrylamide gel electrophoresis as previously described (8). Control noninfected cells are presented in the first lane.

covered after 2 h of exposure to virus (Fig. 2). Polyacrylamide gel electrophoresis analysis of the nuclear HFV proteins revealed the presence of Gag intermediate p56, MA, and Bet but not the Gag precursors. Use of specific antisera confirmed the presence of MA, but no significant amounts of CA or NC were detectable. These experiments with labeled incoming virus clearly demonstrate that Gag proteins rapidly accumulate in the nucleus after infection in the absence of viral protein synthesis.

The detection of mature Gag proteins in our experiments contrasted with the results of others (31). These differences could have been due to the mode of preparation of the nuclear extracts. Therefore, we evaluated the different parameters (NaCl concentration, detergents, and/or DNase I treatment) for the processing of newly synthesized nuclear Gag proteins in productively infected cells. First, nuclear pellets were incubated with DNase I at increasing NaCl concentrations; only uncleaved Gag precursors and Bet proteins were detected at a low NaCl concentration (Fig. 3A). However, higher salt concentrations led to the complete disappearance of uncleaved Gag precursors and Bet proteins and to exclusive recovery of MA and of a p52 protein that appears to be a truncated Bet protein (unpublished data). Second, when nuclei were extracted in the presence of 1 M NaCl, 1% Triton X-100, 0.5% DOC, and 0.05% SDS but without treatment with DNase I, only uncleaved Gag proteins were found. Treatment of the nuclear remnants with DNase I yielded the processed proteins, i.e., essentially MA and p52 Bet (Fig. 3B). However, in nuclei from HFV-infected cells treated with high NaCl concentrations, detergents, and DNase I, only processed Gag antigens were found by immunoprecipitation (Fig. 3C). The presence of uncleaved Gag precursors in the nuclei of infected cells confirms the previous report of Schliephake and Rethwilm (31). However, we show here that incubation with DNase I at a high saline concentration and in the presence of detergents, none of which were used by these authors, allows specific cleavage of Gag proteins. Finally, in order to clarify the role of DNase I, other nucleases, such as micrococcal nuclease, RNase, and the restriction enzymes *Eco*RI and *Xho*I, were tested (data not shown). Nuclei were digested with or without 1% Triton X-100, 0.5% DOC, and 0.05% SDS (Fig. 3D). In the presence of DNase I, Gag precursors were processed to a variable extent depending on the presence or absence of detergents. In contrast, incubation with the other nucleases, even in the presence

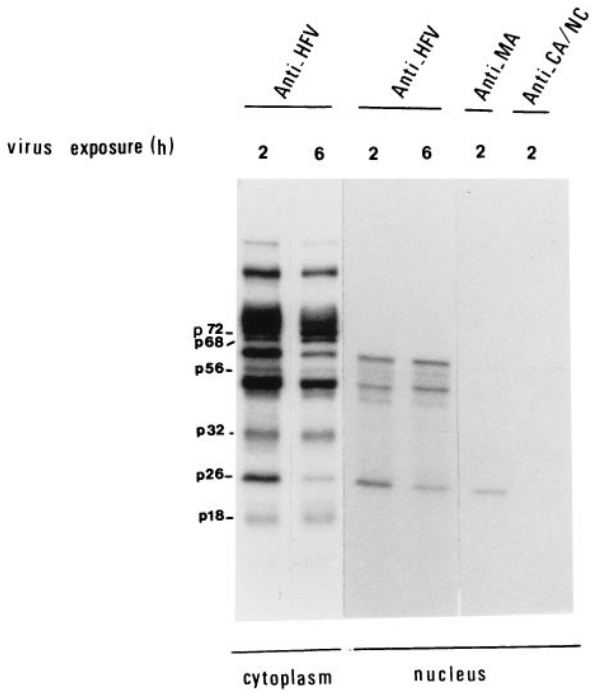

FIG. 2. Subcellular localization and maturation of incoming HFV proteins. U373-MG-infected cells were labeled overnight with a ^{35}S protein-labeling mixture of Met and Cys (150 μCi/ml; specific activity 1,195 Ci/mmol;NEN) in medium without methionine and cysteine but with 5% fetal calf serum. Cells were scraped into medium without serum and lysed by two steps of freeze-thawing. After centrifugation (5 min at 1,800 × *g*), the cell supernatant was used as labeled virus stock. Immunoprecipitation of viral proteins from cytoplasmic and nuclear extracts with anti-whole-virus antiserum or specific Gag antisera was performed after 2 and 6 h, respectively, of exposure to virus at 37°C.

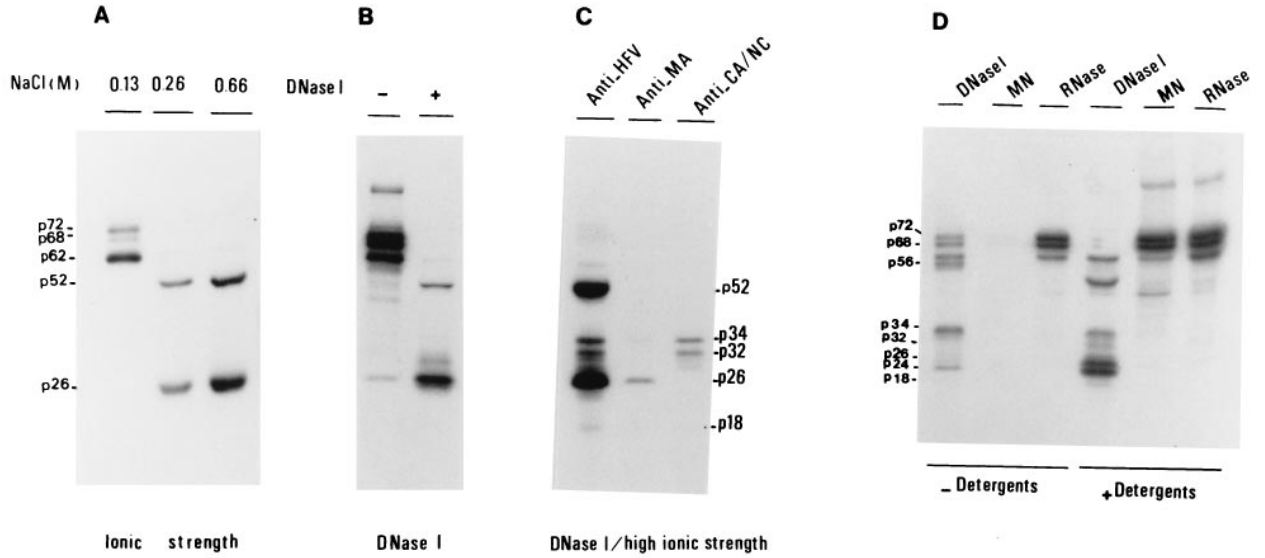

FIG. 3. Effect of ionic strength, DNase I, and detergents on Gag maturation. Nuclear pellets were incubated for 20 min at 20°C in lysis buffer containing 0.05 M Tris (pH 7.4), 0.005 M $MgCl_2$, 1% Triton X-100, 0.5% DOC, 0.05% SDS, and 0.003 M phenylmethyl sulfonyl fluoride with DNase I (A), with DNase I at high NaCl concentrations (B and C), and with different nucleases in the presence or absence of detergents at high NaCl concentrations as follows: DNase I for 20 min at 20°C, micrococcal nuclease (MN 10 μg/ml) for 30 min at 37°C and pancreatic DNase-free RNase (20 μg/ml) for 1 h at 37°C (D). When used, DNase I was at a concentration of 33 μg/ml. Immunoprecipitation was realized with the anti-whole-virus antiserum except in panel C, where Gag-specific antisera were used.

of detergents, never led to any cleavage of viral proteins. All together, our results suggest that Gag processing is not relevant to the nuclease activity of DNase I but could relate to another property of this protein.

A characteristic feature of DNase I, besides its nuclease activity, is its stoichiometric interaction with the actin monomer G-actin (12, 22, 29). This interaction can inhibit actin polymerization and even depolymerize F-actin. To test the hypothesis that cleavage of Gag may relate to a nonnuclease effect of DNase I, we performed immunoprecipitation experiments with cytoplasmic extracts of HFV-infected cells. Strikingly, treatment with DNase, NaCl, and detergents resulted in complete cleavage of the Gag precursor polypeptides (Fig. 4A, lane 2). Remarkably, this cleavage was completely abolished by addition of two distinct antiprotease antisera directed either against recombinant proteins (2) or against a synthetic peptide (provided by J. Gerfaux, Montpellier, France) (Fig. 4A, lane 3). This result could suggest that the highly specific property of

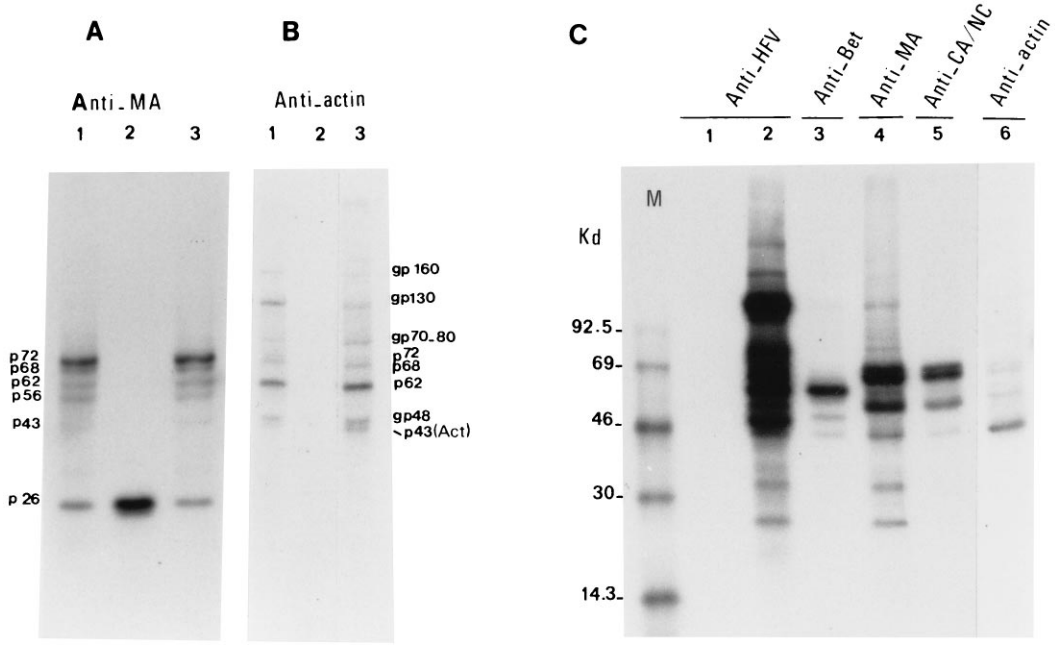

FIG. 4. Cross-immunoprecipitation of viral proteins and actin (A and B). Immunoprecipitation of cytoplasmic extracts was performed with anti-MA antiserum (A) or antiactin antiserum (B). Lanes 1, no DNase I; lanes 2, DNase I; lanes 3, DNase I in the presence of polyclonal anti-HFV protease antiserum prepared in our laboratory. (C) Control noninfected cells (lane 1) and infected cell extracts (lanes 2 to 6) were immunoprecipitated with antisera or antibodies as shown.

DNase I to induce Gag cleavage relates to its ability to interact with actin. The same extracts were immunoprecipitated with a polyclonal antiactin antiserum (Sigma). In addition to actin at 43 kDa, HFV polypeptides were immunoprecipitated, demonstrating that HFV proteins and actin are present in the same complex (Fig. 4B, lane 1). Strikingly, treatment of the same extracts with DNase I abolished not only HFV precipitation but also that of actin (Fig. 4B, lane 2). Interestingly, addition of specific anti-HFV protease antiserum during DNase I treatment led to complete recovery of all the proteins previously revealed by the antiactin antiserum (Fig. 4B, lane 3). Moreover, with the antiactin antiserum as well as with at least four different anti-HFV antisera prepared in different fashions (from bacterial fusion proteins or synthetic peptides) and directed against whole virus, Bet, MA, CA, and NC, actin was immunoprecipitated, favoring physical contact between HFV polypeptides and the actin cytoskeleton (Fig. 4C, lanes 1 to 6).

The observed low-level processing of Gag precursors in the different cellular compartments of HFV-infected cells as well as in free viral particles is a characteristic feature of HFV. Several recent reports which focus on the Pol proteins of HFV and on viral protease activity tend to indicate a normal processing of the Pol precursor by viral protease leading to active DNA polymerase, RNase H, and integrase viral proteins (15, 16, 26, 28). In this study, we produced the first evidence for a complete processing of Gag precursors by the HFV protease.

The accumulation of uncleaved Gag proteins in the nuclei of infected cells might help to stabilize unintegrated forms of viral DNA. Unlike in HIV, in HFV the nuclear localization signals, required for the nuclear transport of Gag proteins, were shown to be provided by NC but not by MA (5–7, 31, 33). We have shown using labeled viral preparations that the nuclear transport of Gag polypeptides can occur in the absence of viral protein synthesis. The previous report of Schliephake and Rethwilm tended to exclude this possibility (31). The high sensitivity of our approach using labeled virions could explain the difference.

While we have identified two situations where Gag processing by the viral protease is observed, how these relate to each other is unclear. In our experiments, MA was the most abundant and sometimes the only mature Gag protein detected in cytoplasmic or nuclear extracts treated with DNase I. Whether the abundance of MA observed here was due to its stabilization by viral or cellular proteins, or by viral RNA or DNA, remains unknown. The variable but always low abundance of NC and CA in processed extracts likely reflects their degradation.

In this study, several facts support the idea of a direct interaction between viral proteins and actin microfilaments: (i) the coimmunoprecipitation of actin and viral proteins, even from cell extracts prepared in the presence of reducing agents or of SDS; (ii) the unexpected coprecipitation of different viral proteins with specific antisera raised against a single recombinant protein (e.g., Bet precipitated by anti-MA antiserum [Fig. 4A]), and (iii) the onset of viral protease activity following DNase I treatment. The fact that the immunoprecipitation of actin is abolished by treatment with DNase I and recovered by adding the antiprotease antiserum points to actin as a substrate for HFV protease, as previously demonstrated for HIV (10, 34–36). Interaction between HFV proteins and actin may constitute one more example of the numerous observed interactions between viruses and subcellular structures (4, 9, 20, 21, 23, 27, 32). We hypothesize that the association of Gag precursors with actin microfilaments might prevent recognition of specific cleavage sites by viral protease and thus explain the abundance of these unprocessed precursors in HFV-infected cells.

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