VIROLOGY 193, 981–985 (1993)

Identification of a Region in the Pr55^{gag}-Polyprotein Essential for HIV-1 Particle Formation

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Received September 2, 1992; accepted December 4, 1992

The pr55^{gag} polyprotein of HIV-1 plays a critical role in the formation of immature virus particles in the cell and during the budding process. We investigated the influence of amino acid substitutions in the p24CA- region of the *gag* polyprotein on the viral assembly process. Deletion of the amino acids 341–352 in the carboxy terminal part of the p24CA resulted in a loss of the capacity of the *gag* polyprotein to form virus-like particles when expressed in eucaryotic cells by recombinant vaccinia virus. In further experiments it turned out that the amino acids 341–346 and 350–352 are important for the ability of the pr55^{gag} to form virus-like particles. Because these stretches are conserved among HIV-1, HIV-2, SIV, and FIV, we conclude that these amino acids form a domain highly important for the assembly of these lentiviruses.

The assembly process of HIV-1 is a complex multistep process that is so far not well understood. The known elements required for particle formation are localized in the major structural precursor protein pr55^{gag} of the virus. This polyprotein which is myristoylated cotranslationally at the aminoterminus comprises the p17MA, the p24CA, the p7NC, the p6LI, and two small proteins p2 and p1 (*1–4*). The pr55^{gag} molecules are synthesized on free ribosomes and accumulate at the inner side of the plasma membrane where they form the immature viral particle that is released from the cell by budding. Afterward, the *gag* proteins of the mature viral particle are formed by the activated viral protease in a processing reaction (*3*). Several regions of the protein active in the assembly process have been identified so far. In previous experiments it was shown that myristoylation is essential for targeting the precursor protein to the plasma membrane (*5*). A zinc-finger domain located in the p7NC is responsible for the interaction with the RNA genome and its packaging into the particle (*6*). The p6LI seems to be necessary for the closure of the particle during the budding process (*7, 8*). Experiments with carboxy terminally truncated pr55^{gag} polyproteins expressed by different transient expression systems in eucaryotic cells confirmed the role of the p15 region in the process of viral particle assembly (*9, 10*).

Furthermore protein–protein interactions have been estimated to be essential for accumulation of the pr55^{gag} molecules at the plasma membrane and initial budding events. We investigated the effect of mutations in the p24CA possibly involved in mediating such interactions as shown in previous experiments (Niedrig *et al.*, manuscript in preparation). Here peptides corresponding to amino acids 340–350 of the pr55^{gag} specifically inhibited the formation and release of infectious viral particles. One hypothesis for this inhibitory activity is that alterations in the density of the accumulated pr55^{gag} polyprotein molecules result in incorrect formation of budding structures, followed by a block in late steps of viral maturation. To investigate the role of that region in the assembly process, we decided to exchange the p24CA-specific amino acids for alanine and expressed the resulting mutant proteins in eucaryotic cells by recombinant vaccinia viruses (Fig. 1). In the absence of *env* proteins or the viral enzymes, the *gag* proteins of HIV-1 are known to form retroviral core-like particles in a manner very similar to the viral assembly process when expressed in eucaryotic cells (*5, 9–11*). The influence of alterations in the amino acid sequence on that process should provide information about residues with side chains critical for the budding process. Additionally a mutant protein was created with a complete deletion of amino acids 340–350 (Fig. 1).

For this purpose the 5′-region from nucleotide −6 to +1004 and the 3′-region from nucleotide +1065 to +1651 of the *gag* protein coding sequence of HIV-1 strain BH102 (*12*) were separately amplified in a PCR. Thereby a *Bam*HI site was introduced 5′ of the ATG codon and *Bfr*I and *Xho*I sites were introduced at the 3′-end of the amplified DNA of the 5′-region. In the 3′-

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0042-6822/93 $5.00

		340 352		particle formation: gradient	particle formation: EM
wild-type	ALGP	AATLEEMMTACQG	VG	+	+
pr55Gag/1	ALGP	AAAAEEMMTACQG	VG	-	-
pr55Gag/4	ALGP	AATLAAAMTACQG	VG	-	-
pr55Gag/7	ALGP	AATLEEMAAACQG	VG	+	+
pr55Gag/10	ALGP	AATLEEMMTAAAA	VG	+	altered
pr55Gag/D	ALGP	----------QG	VG	-	-

FIG. 1. Amino acid sequences of wild-type and mutant pr55^{gag} in the investigated p24 region. The amino acids 341 to 352 were exchanged in tripletts for alanine or residues 340 to 350 were completely deleted. The mutations were generated as described in the text. The capacity to form core-like particles was assayed by sucrose gradient centrifugation and electron microscopy (EM). +, particles detected; −, no particle formation. In p55Gag/10 the morphology of the particles was altered.

part *Xho*I and *Sma*I sites were generated at the 5′-end and a *Sal*I/*Hin*cII site at the 3′-end. The 5′-fragment was inserted into the plasmid plin8p55VIII-B4 after digestion with *Bam*HI and *Xho*I (plasmid plin8p55/9; all enzymes were purchased from Boehringer-Mannheim, Mannheim, Germany). Subsequently the 3′-fragment was inserted into this plasmid via the *Xho*I and *Hin*cII sites to give plin8p55-V. This plasmid contains the pr55^{gag} sequence in which the amino acids 335 to 354 are replaced by residues L and E. Synthetic oligonucleotides were inserted in the *Bfr*I/*Sma*I-digested plasmid plin8p55-V to give the mutant pr55^{gag} sequences plin8p55/1, plin8p55/4, plin8p55/7, and plin8p55/10 coding for the mutant proteins p55Gag/1, p55Gag/4, p55Gag/7, and p55Gag/10. In these mutants the amino acids 341–343 (p55Gag/1), 344–346 (p55Gag/4), 347–349 (p55Gag/7), and 350–352 (p55Gag/10) were substituted by alanine (Fig. 1). All constructs were verified by DNA sequencing using the dideoxynucleotide method. Alanine is probably the most neutral substitute and is not expected to induce major structural effects by itself. This is due to its small size and the lack of functionally active side chains. In the plasmid plin8p55/D the amino acids 340 to 350 were deleted. The mutant pr55^{gag}coding sequences were inserted in the vaccinia virus transfervector pAVBII (*13*). Recombinant vaccinia viruses were obtained by transfection/infection as described by Mackett (*14*). Briefly, HAT-selected thymidine kinase-positive vaccinia virus strain vTT was used to infect 143B cells. The infected cells were cotransfected with 2 μg of the vaccinia virus transfer vector DNA and screened for recombinant viruses by BrdU selection. From all constructs proteins were expressed at comparable levels of about 60–140 pg of pr55^{gag} per 10^7 cells as quantified by a commercial p24 capture assay (Abbott, Wiesbaden, Germany). All mutants expressed a *gag* protein of the appropriate molecular weight as shown by Western blot analysis. As control a vaccinia virus expressing the HIV protease fused in-frame with the pr55 was included. This allows the identification of possible cleavage products of the mutant proteins in addition to the complete pr55^{gag}. No degradational patterns comparable with the authentic processed forms were observed. The mutant proteins differed only slightly in their electrophoretic mobility due to different losses of molecular weight. Only p55Gag/4 and p55Gag/D reproducibly showed a stronger shift in electrophoretic mobility (Fig. 2). In the case of p55Gag/4 this may be caused by the loss of two negatively charged amino acids in addition to the decrease in molecular weight.

After two cycles of plaque purification the particle-forming capacity of the pr55^{gag} polyproteins was investigated by centrifugation on a 10–65% sucrose gradient. SW480 cells (10^7) were infected with the recombinant vaccinia viruses at a m.o.i. of 1. Three to four days p.i. the supernatants were loaded on gradients and centrifuged at 220.000 *g* for 1.5 hr. Vaccinia viruses expressing a myristoylation-deficient mutant and the original pr55^{gag} were included as controls (Fig. 3A). Twenty samples of 500 μl were taken from the top of the gradient and diluted with water (total volume 1.5 ml), and particular structures were pelleted by centrifugation (Kontron A8.24 rotor, 20.000 rpm, 90 min). The pellet was lyophylized and resolved in SDS containing solubilization buffer (according to *15*). After separation on a 17.5% SDS–PAGE gel and transfer to nitrocellulose membranes by Western blotting the pr55^{gag} was detected with the monoclonal antibody 13/5 recognizing amino acids 133 to 158 and antibody 16/4/2 specific for amino acids 307–336 of the pr55^{gag}, respectively (*16*). The mutants p55Gag/7 and p55Gag/10 showed a maximum of antigen in sucrose gradients at a density around 1.15 to 1.18 g/cm^3, typical for HIV-1 core-like particles (Fig. 3B) (*9, 11, 13*). Recombinant vaccinia virus expressing p55Gag/7 produced nearly twice the amount of antigen as the other mutants when assayed by a commercial p24 capture assay (data not

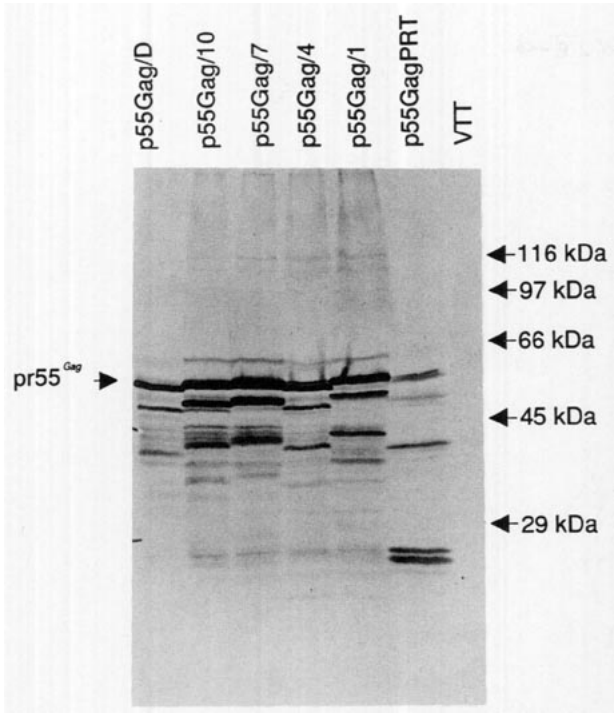

FIG. 2. Expression of the pr55^{gag} constructs by recombinant vaccinia viruses. SW480 cells were infected with recombinant vaccinia viruses, harvested, and separated on a 17.5% SDS–PAGE gel as described in the text. The expressed proteins were detected with the *gag*-specific monoclonal antibodies 13/5 and 16/4/2 recognizing epitopes in the p17MA and the p24CA. The mutant proteins differ slightly in their electrophoretic mobility due to differences in molecular weight between the alanines and the replaced amino acids. The additional loss of two negative-charged residues in p55Gag/4 causes a greater mobility shift. The apparently reduced molecular weight of p55Gag/D is explained by the deletion of amino acids 340–350. To identify the native pr55^{gag} and putative cleavage products, an in-frame expression clone of pr55^{gag} and the viral protease (p55GagPRT) were included. The cleavage products of pr55^{gag}, p41, and p24/25, respectively, are clearly visible. A nonrecombinant vaccinia virus of strain vTT is shown at the right. Positions of the molecular weight markers are as indicated.

shown), leading to a smear in the gradient due to overload with antigen (Fig. 3B). Using the deletion mutant p55Gag/D and mutants p55Gag/1 no peak could be detected. Very small amounts of the mutant pr55^{gag} proteins were frequently present in most gradient fractions from about 1.05 to 1.20 g/cm^3 of all mutants. With p55Gag/4, more antigen was occasionally found in the gradient at a density of about 1.08 g/cm^3. According to our electron microscopical data this antigen did not represent virus-like particular structures (data not shown). Instead it could represent altered forms of pr55^{gag} bound to the membranes and released in vesicular forms during cell lysis by vaccinia virus. Such structures could also be observed by electron microscopy (data not shown). The double band observed in the analysis of p55Gag/D on Western blots (Fig. 3B) may indicate that this protein has become susceptible to partial cleavage by cellular proteases. This can be due to conformational changes of the protein or to introduction of a new cleavage site created by the deletion.

To confirm the results of the sucrose density centrifugation, we determined the ability of the mutant pr55^{gag} polyproteins to form retroviral core-like particles by ultrathin section electronmicroscopy. SW480 cells were infected with the recombinant vaccinia viruses, as described above, harvested 3–5 days p.i. (approximately 50% lysis), and fixed with 2.5% glutaraldehyde. As controls uninfected cells or cells infected with a wild-type pr55^{gag} containing vaccinia virus were included. After staining with uranyl acetate the probes were examined in the electronmicroscope (Siemens Elmiskop 101 operated at 80 kV). In cells infected with the mutant p55Gag/7 retroviral core-like particles could be easily detected. They showed the typical doughnut-shaped structure consisting of an electron-dense ring of *gag* protein surrounding an electron lucent lumen in the particle. No particles were found to be released from cells expressing p55Gag/1, p55Gag/4, and p55Gag/D as expected from the prior experiments. Here, osmiophilic protein complexes accumulated at the inner side of the plasma membrane probably consisting of mutant pr55^{gag} polyproteins (data not shown). Vesicular structures could be observed in these preparations containing such complexes and most likely represent protein associated with fragments of the plasma membrane caused by cell lysis by the vaccinia virus. In cells infected with recombinant vaccinia viruses expressing p55Gag/10, vesicles of a different but defined shape were frequently found (data not shown). These vesicles could not be detected in the vTT-infected controls. Therefore these structures may represent *gag* containing particles of a different type. They were surrounded by a membrane and a protein shell of undefined fine structure and contained osmiophilic protein complexes in the center. That these complexes consist of p24CA is very unlikely since no proteolytic processing takes place as can be told from the protein pattern in Western blot analysis of the particles in the gradient (Fig. 3B). However, none of these structures showed the characteristic morphology of immature core-like particles.

In conclusion, we could show that deletion of amino acids 340–350 or mutation of amino acids 341–346, respectively, destroys the capacity of the pr55^{gag} to form retroviral core-like particles. Exchange of amino acids 350–352 causes an alteration of the assembly pathway, resulting in particular structures different from immature core particles.

The observation that the altered pr55^{gag} polyproteins are still synthesized, transported to, and inserted in the plasma membrane suggests an interruption of the assembly process at a late stage. While in the case of p55Gag/D an overall conformational effect on the pr55^{gag} structure by the deletion of 11 residues cannot be excluded, the exchange of single amino acid resi-

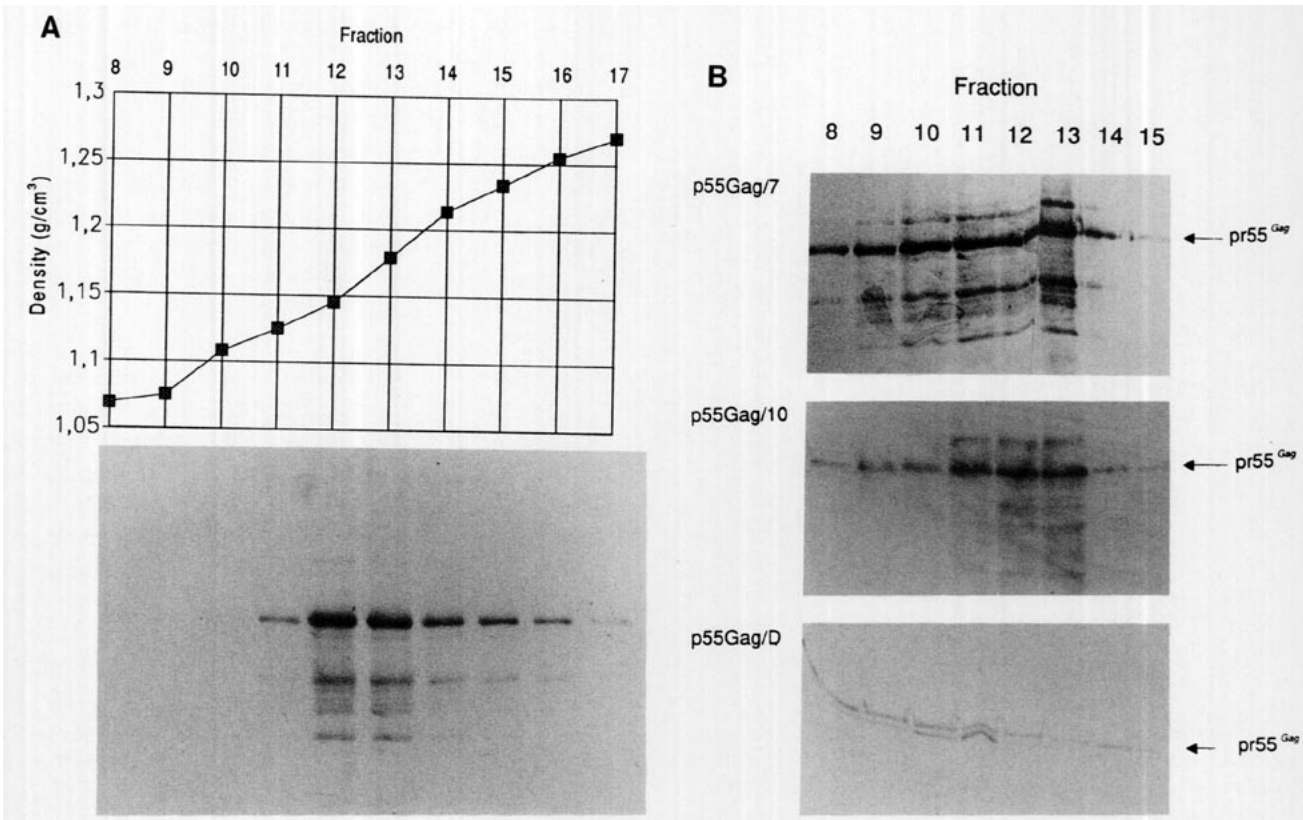

FIG. 3. Investigation of the particle-forming capacity of the mutant proteins by sucrose density centrifugation. The supernatants of the infected cells were analyzed on the sucrose gradient as described in the text and 20 fractions were collected. (A) Antigenic peaks were observed at a density of about 1.15 to 1.18 g/cm³ (fractions 12 + 13) following analysis of the supernatants of cells infected with vaccinia viruses expressing p55Gag wild-type protein. (B) The antigen-positive fractions reaching from a density of 1.05 to 1.21 g/cm³ after separation on a 17.5% SDS–PAGE gel and detection with two *gag*-specific monoclonal antibodies are shown. Peak formation was observed with p55Gag/7 and p55Gag/10 only. The highest levels of expression were seen with p55Gag/7, leading to an overload and smear of the gradient. Mutant p55Gag/D as well as p55Gag/1 and p55Gag/4 (not shown) showed no defined peak.

dues for alanine is likely to represent only minor structural alterations and should exert no major effect on the protein conformation. There are different possible explanations for the observation that the mutated *gag* molecules are incapable of wrapping the cell membrane around the core complex. The CA regions of retroviral *gag* proteins have been shown to be important for the assembly of these viruses (*17–19*). The favored hypothesis is that they mediate the interactions between the *gag* molecules during the aggregation at the inner side of the plasma membrane (*19, 20*). The amino acid alterations may reduce the ability of the molecules to exert protein–protein interactions. This might in turn lead to a lower packaging density at the membrane. As a result, local physical forces needed to round up the membrane could be too weak. On the other hand the mutants could represent flexibility mutants. It has been hypothesized that after initial insertion of the *gag* proteins into the membrane, conformational changes may occur, leading to exposure of more hydrophobic residues on the surface of the protein (*20, 21*). Consequently, the proteins gain the ability to wrap the membrane around the budding complex. It has been suggested that this kind of mutant shows a phenotype in which the proteins are correctly inserted into the lipid bilayer but fail to draw it around (*20*). This correlates with our observations made by analysis of p55Gag/1 and p55Gag/4, where no particles could be observed in the electron microscope and in the sucrose gradient. If this hypothesis is conferred to p55Gag/10, it seems that conformational changes occur and particles are still going to be released. But structural alterations might occur in a different way. Therefore, amino acids 350 and 351 may not be essential for the budding process itself but for the formation of correctly structured particles. This function must not necessarily be exerted later in the assembly pathway.

The investigated domain contains the amino acid sequences TLEE (position 342–345) and CQ (350–351), representing highly conserved residues not only between different HIV-1 isolates but also between HIV-1, HIV-2, SIV, and FIV (*22, 23*). In all these viruses the sequence is located in the carboxy terminal part of the CA protein (Fig. 4). With respect to the low fidelity of the retroviral reverse transcriptase (*24*), the conservation of sequence and location indicates a potential similar function of the investigated region in these lentiviruses.

	340	351
HIV-1	AA**TLEE**MMTA**CQ**	
	331	342
HIV-2/SIV	NP**TLEE**MLTA**CQ**	
	337	348
FIV	ES**TLEE**KLRA**CQ**	

FIG. 4. Sequence comparison of the carboxy terminal part of the capsid proteins from HIV-1/2, SIV, and FIV. The sequence TLEEXXXXCQ is highly conserved in these viruses. The numbers of the first and last amino acids are given with respect to the first codon of the *gag* polyprotein of these viruses. Mutations of the amino acids TLEE (underlined) abolished the capacity of the $pr55^{gag}$ of HIV-1 to form core-like particles (p55Gag/1 and p55Gag/4). Mutation of C and Q in p55Gag/10 leads to formation of particles with a different morphology. Alterations of amino acids 347–349 (p55Gag/7) had no impact on the assembly process.

As suggested by our data the described domain may play a role in two different steps of the assembly pathway. Additionally, an equally located sequence has been identified in the *gag* proteins of Rous sarcoma virus. Wills and co-workers suggested a role for this domain in mediating protein–protein interactions of the *gag* proteins during their accumulation at the plasma membrane (*20*). In contrast, the whole CA region of Moloney murine leukemia virus is sensitive to mutations (*25*). Some authors suggested that the amino terminal part of this region may be more important in this virus but it remains unclear because the carboxy terminal substitution made in those experiments was conservative regarding the nature of the amino acids. On the other hand, this specific insertion might have been located at a less important site (*17*). We therefore suggest that the carboxy terminal region of the CA proteins of lentiviruses and C-type retroviruses contains a domain that plays an essential role in the assembly process. With respect to therapy of HIV infection, the assembly-defective *gag* proteins could be useful in negative complementation attempts in therapy as previously discussed for the *gag* protein and the regulatory proteins *rev* and *tat* (*26–28*). Further studies to elucidate the exact functional significance of this domain are currently under way.

ACKNOWLEDGMENTS

We are grateful to Holger Fließbach for helpful discussion and thank Margret Brand and Ilka Dürr for technical assistance. The plasmid pAVBII was provided by Dr. v. Brunn. This work was supported by Grant No. 5 of the Forschungsverbundprojekt FVP 188.

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