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Functional domains in the feline immunodeficiency virus nucleocapsid protein

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Abstract

Retroviral nucleocapsid (NC) proteins are small Gag-derived products containing one or two zinc finger motifs that mediate genomic RNA packaging into virions. In this study, we addressed the role of the feline immunodeficiency virus (FIV) NC protein in the late stages of virus replication by analyzing the assembly phenotype of FIV NC mutant viruses and the RNA binding activity of a panel of recombinant FIV NC mutant proteins. Substitution of serine for the first cysteine residue in the NC proximal zinc finger was sufficient to impair both virion assembly and genomic RNA binding. A similar defective phenotype with respect to particle formation and RNA binding was observed when the basic residues Lys28 and Lys29 in the region connecting both zinc fingers were replaced by alanine. In contrast, mutation of the first cysteine residue in the distal zinc finger had no effect on virion production and allowed substantial RNA binding activity of the mutant NC protein. Moreover, this NC mutant virus exhibited wild-type replication kinetics in the feline MYA-1 T-cell line. Interestingly, amino acid substitutions disrupting the highly conserved PSAP and LLDL motifs present in the C-terminus of the FIV NC abrogated virion formation without affecting the NC RNA binding activity. Our results indicate that the proximal zinc finger of the FIV NC is more important for virion production and genomic RNA binding than the distal motif. In addition, this study suggests that assembly domains in the FIV NC C-terminus may be functionally equivalent to those present in the p6 domain of the Gag polyprotein of primate lentiviruses.

Keywords: Feline immunodeficiency virus; Nucleocapsid protein; Virion assembly; Genomic RNA binding; Retroviruses

Introduction

The *gag* product of retroviruses is a polyprotein necessary for the assembly, budding, and release of virions from infected cells (reviewed in Hunter, 1994). During or shortly after virion release from the cell surface, the Gag polyprotein is cleaved by the viral protease to generate the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins (Hunter, 1994). The MA protein is closely associated with the viral membrane, whereas the CA protein is the major component of the electron dense core of the mature virions (Hunter, 1994). The NC protein is located within the CA-derived core and coats two molecules of the viral genomic RNA. The selective encapsidation of the full-length unspliced viral genomic RNA from a pool of cellular and viral RNAs is an essential stage in the life cycle of all retroviruses which requires the recognition by the unprocessed Gag polyprotein of an RNA sequence, termed encapsidation signal (E) or packaging signal (ψ), located at the 5′ end of the genome and often extending into the *gag* gene (Jewell and Mansky, 2000). The NC domain of the Gag precursor is crucial for the encapsidation of the viral genome in several retroviruses (reviewed in Darlix et al., 1995). All retroviral NC proteins exhibit a high content of basic residues and, with the exception of spumaviruses, contain one or two copies of a zinc-binding motif with the sequence Cys-X2-Cys-X4-His-X4-Cys that is similar to those found in many DNA-binding proteins (Jewell and Mansky, 2000). Amino acid substitutions targeting the basic residues as well as the zinc-binding motifs of the NC protein interfere with RNA binding in vitro (Dannull et al., 1994; Schmalzbauer et al., 1996) and with RNA packaging in vivo (Akahata et al., 2003; Aldovini and Young, 1990; Aronoff et al., 1993; Dorfman et al., 1993; Dupraz et al., 1990; Gorelick et al., 1988; 1999; Méric and Goff, 1989; Méric et al., 1988; Poon et al., 1996). In addition to its role in

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genomic RNA packaging, it has been reported that the NC domain of the Gag precursor promotes the annealing of the primer tRNA to the genomic primer-binding site and facilitates genomic RNA dimerization (Darlix et al., 1990; Prats et al., 1988). Moreover, evidence consistent with a role for the mature NC protein during the early steps of the virus life cycle has been presented. In this regard, the NC participates in the DNA strand transfers that occur during reverse transcription (Allain et al., 1994; Tsuchihashi and Brown, 1994).

Several studies have addressed whether the integrity of the NC domain of the retroviral Gag precursor is necessary for efficient particle assembly and release. It has been shown that the simultaneous substitution of functionally relevant amino acids in both zinc-binding domains of the human immunodeficiency virus type 1 (HIV-1) NC significantly reduces virion release (Dorfman et al., 1993). A similar impairment in HIV-1 Gag particle formation was observed when mutations were introduced into the N-terminal basic domain and in the basic linker region between the zinc fingers of the HIV-1 NC protein (Cimarelli et al., 2000; Dawson and Yu, 1998; Sandefur et al., 2000; Zhang and Barklis, 1997). Moreover, when a panel of HIV-1 Gag deletion mutants was expressed in bacteria and evaluated for their ability to associate with full-length Gag in vitro, the NC protein exhibited an association activity comparable to that of the complete Gag polyprotein (Burniston et al., 1999). Taken together, these results underscore the importance of the NC domain in Gag–Gag oligomerization and assembly.

Compared with the numerous reports describing the functions of the HIV-1 NC protein, little is known about the role that this protein plays in the life cycle of non-primate lentiviruses. Feline immunodeficiency virus (FIV) is a lentivirus that causes an AIDS-like syndrome in its natural host, the domestic cat (Barlough et al., 1991). Given that the FIV-cat system is widely used as an animal model for both HIV vaccines and antiviral strategies (Bendinelli et al., 1995) and that the retroviral NC protein is recognized as a target for therapeutic interventions, it is therefore important to characterize the molecular determinants involved in the FIV NC function. This information will not only contribute to our understanding of the role played by the viral proteins shared by primate and non-primate lentiviruses, but will also prove useful for the rational design of antiviral strategies directed against HIV and animal lentivirus infections. In the present study, we performed a site-directed mutagenesis analysis of the FIV NC protein to identify functional domains within this Gag product. The NC mutants were expressed in the context of FIV proviral constructs and examined for their ability to assemble into virions in transfected feline cells. In addition, recombinant FIV NC mutant proteins were tested for viral RNA binding activity in Northwestern assays. Our results demonstrate that the proximal zinc finger plays a more prominent role than the distal one not only in viral RNA binding but in virion assembly as well. Moreover, we show here that domains within the small Gag peptide C-terminal to the FIV NC are necessary for efficient particle production.

Results

Mutagenesis of the NC domain of the FIV Gag precursor

To identify functional domains within the FIV NC protein, we performed a site-directed mutagenesis analysis of the genomic region encoding this Gag product. The NC protein consists of 84 amino acids if considering the C-terminal Gag polypeptide extending from the viral protease (PR) cleavage site at the CA/NC boundary (Gag residues Gln366–Val367) to the stop codon for the Gag precursor (Elder et al., 1993). However, the ion spray mass spectrometry of FIV proteins has revealed that the major form of the NC in virions has a molecular mass of 7.1 kDa instead of the predicted 9.1 kDa (Elder et al., 1993). Based on this evidence, it has been proposed that there is an additional cleavage site within the FIV NC protein between residues Met66 and Gln67 that would release a small C-terminal Gag peptide (Elder et al., 1993) (Fig. 1).

The specific regions in the FIV NC domain that were targeted for mutagenesis are shown in Fig. 1. Mutations were designed to study structural features in the FIV NC that may be important for NC functions. The first cysteine in each of the two Cys–His boxes was changed to serine (mutations C11S and C30S). Serine was chosen as the replacement because it only differs from cysteine by the substitution of an hydroxyl group for a sulfhydryl group. In addition, two highly conserved basic residues located between the two zinc finger motifs (lysines 28 and 29), as well as the conserved glutamine 45 and asparagine 47 present at positions C-terminal to the second zinc finger motif, were replaced by alanine (mutations K28A/K29A and Q45A/N47A). Furthermore, to study the role in the FIV Gag functions played by the small C-terminal peptide that has been proposed to result from cleavage at FIV NC Met66 (Elder et al., 1993), we introduced within this region three double amino acid substitutions (mutations P72A/P75A, E78A/E79A, and L81A/L84S) that affect the PSAP, EE, and LLDL motifs that are highly conserved among FIV isolates (Fig. 1). The P72A/P75A and L81A/L84S mutations introduce conservative amino acid substitutions in the overlapping *pol* reading frame. However, these changes lie outside of the PR-coding region (Fig. 1). All these mutations in the FIV NC domain were introduced into the proviral DNA of FIV-14 and the phenotype of the resulting mutant viruses was analyzed.

Effect of NC mutations on FIV particle assembly and release

We first studied the ability of the FIV NC mutants to assemble into virions. To this end, Crandell feline kidney (CrFK) cells were transfected in parallel with the wild-type

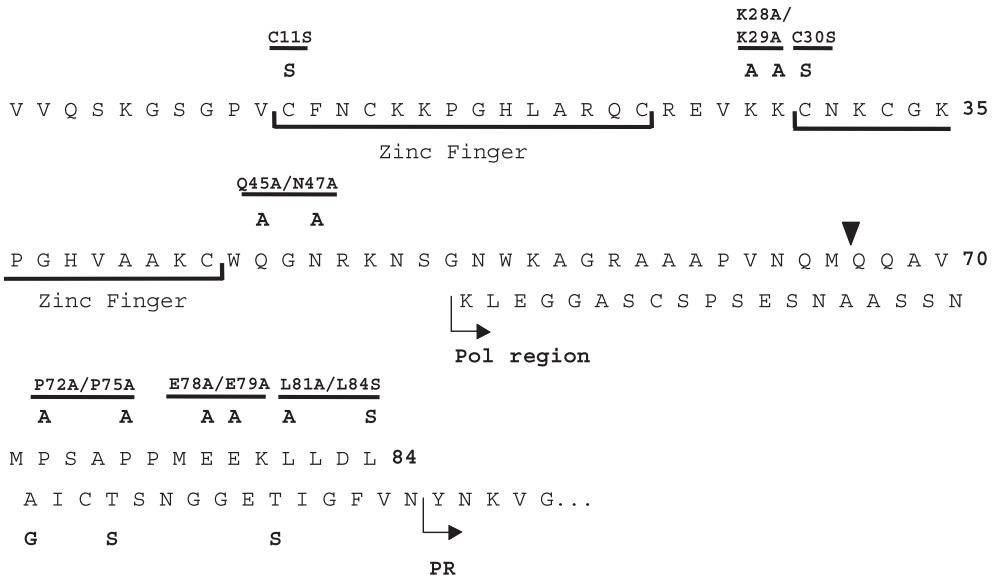

Fig. 1. Mutagenesis of the NC domain of the FIV Gag polyprotein. The amino acid sequence of the Petaluma FIV-14 NC is shown with the overlapping *pol* open reading frame indicated below the Gag sequence. The arrowhead denotes the cleavage site between NC residues Met66 and Gln67, which has been proposed to result in the formation of a small peptide derived from the FIV Gag C-terminus. The single and double amino acid substitutions that were introduced into the FIV Gag region spanning the NC domain and the C-terminal Gag-derived peptide are shown above the sequence. Amino acids in boldface letters below the Pol sequence correspond to conservative changes introduced into this region because of the P72A/P75A and L81A/L84S mutations in the Gag C-terminus. Leucine at position 84 was replaced by serine to avoid the introduction of a nonconservative substitution in the *pol* reading frame.

or the mutant FIV proviral DNAs, and both the cell and virion lysates were assayed for the presence of the FIV Gag and CA proteins by Western blotting. In all but one of the mutants, the levels of Gag expression and processing were similar to those of the wild-type virus (Fig. 2A). The sole exception was the E78A/E79A mutant in which Gag processing was severely impaired (Fig. 2A). When the particulate fraction purified from the culture medium of the transfected cells was analyzed, we found that mutations C11S, K28A/K29A, P72A/P75A, and L81A/L84S signifi-

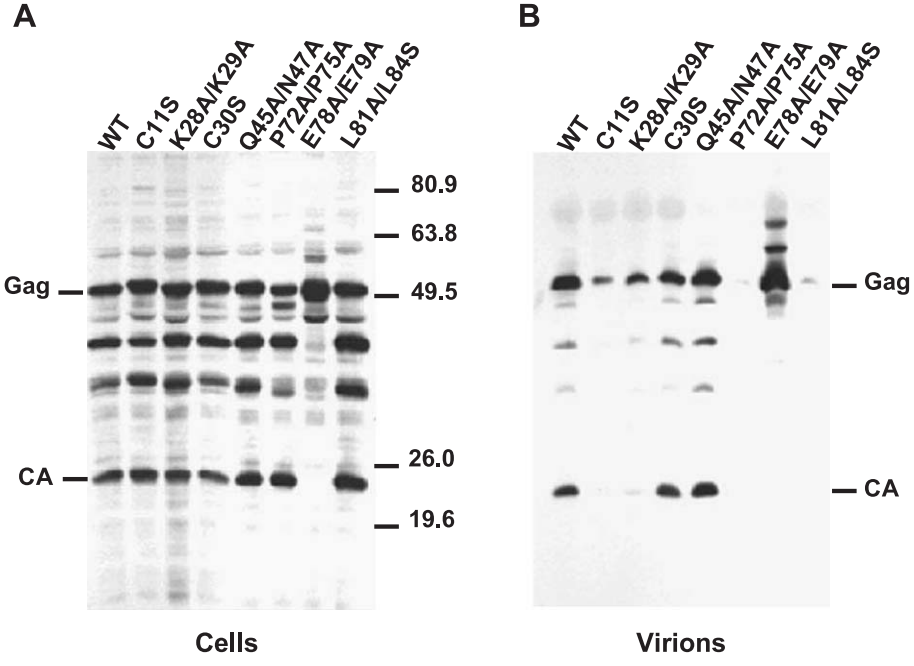

Fig. 2. Effect of mutations in the FIV NC domain on Gag expression and particle formation. CrFK cells were transfected with wild-type (wt) FIV-14 or the NC mutant proviral clones. At 48 h post-transfection, viral proteins from cell lysates (A) or virions (B) were transferred to nitrocellulose membranes and probed with a monoclonal anti-FIV CA antibody. The mobilities of the Gag precursor and CA protein are shown, as are the positions of the molecular weight standards.

cantly reduced virion production when compared to wild-type FIV (Fig. 2B). The most drastic effect was observed for the P72A/P75A and L81A/L84S mutants in which particle-associated Gag and CA proteins were almost undetectable. In contrast, the C30S, Q45A/N47A, and E78A/E79A mutants were assembly-competent and produced virions with an efficiency similar to that of wild-type FIV (Fig. 2B). However, only the FIV Gag polypeptide was detected in the E78A/E79A mutant virions, which correlates with the defect in Gag processing observed in cells expressing this NC mutant. Because the E78A/E79A mutation does not affect the overlapping *pol* reading frame, it is likely that the defect in Gag processing caused by this mutation is due to an altered Gag conformation which conceals the cleavage sites of the Gag polyprotein. Alternatively, this processing defect may reflect an impaired ability of the E78A/E79A Gag mutant to associate with the Gag–Pol polyprotein, which would result in low levels of protease activity in budding virions.

Effect of NC mutations on viral RNA binding

We next asked whether the amino acid substitutions introduced into the FIV NC domain affected its ability to interact with the viral genomic RNA. Because several of the NC mutations impaired particle assembly, it was not possible to determine the efficiency of genomic RNA packaging into virions for all the NC mutants. Therefore, we investigated the ability of recombinant mutant NC proteins to bind viral RNA in vitro. It has been previously demonstrated that labeled RNAs containing the E signal of the HIV-1 genome specifically bind to bacterially expressed Gag or NC proteins immobilized on nitrocellulose membranes (Luban and Goff, 1991; Schmalzbauer et al., 1996). We therefore made use of a similar Northwestern assay to analyze the viral RNA binding capacity of the FIV NC mutant proteins. To this end, wild-type and mutant NC proteins were expressed in bacteria as glutathione *S*-transferase (GST) fusion proteins resolved on 0.1% sodium dodecyl sulfate (SDS)-10% polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were incubated with an uniformly ^{35}S-labeled RNA probe synthesized by in vitro transcription. This RNA probe corresponds to the 5′ end region of the FIV-14 genome comprised between nucleotides 216 and 947 and contains the FIV E signal that has recently been characterized (Kemler et al., 2002). As shown in Fig. 3A, the Q45A/N47A, P72A/P75A, E78A/E79A, and L81A/L84S NC

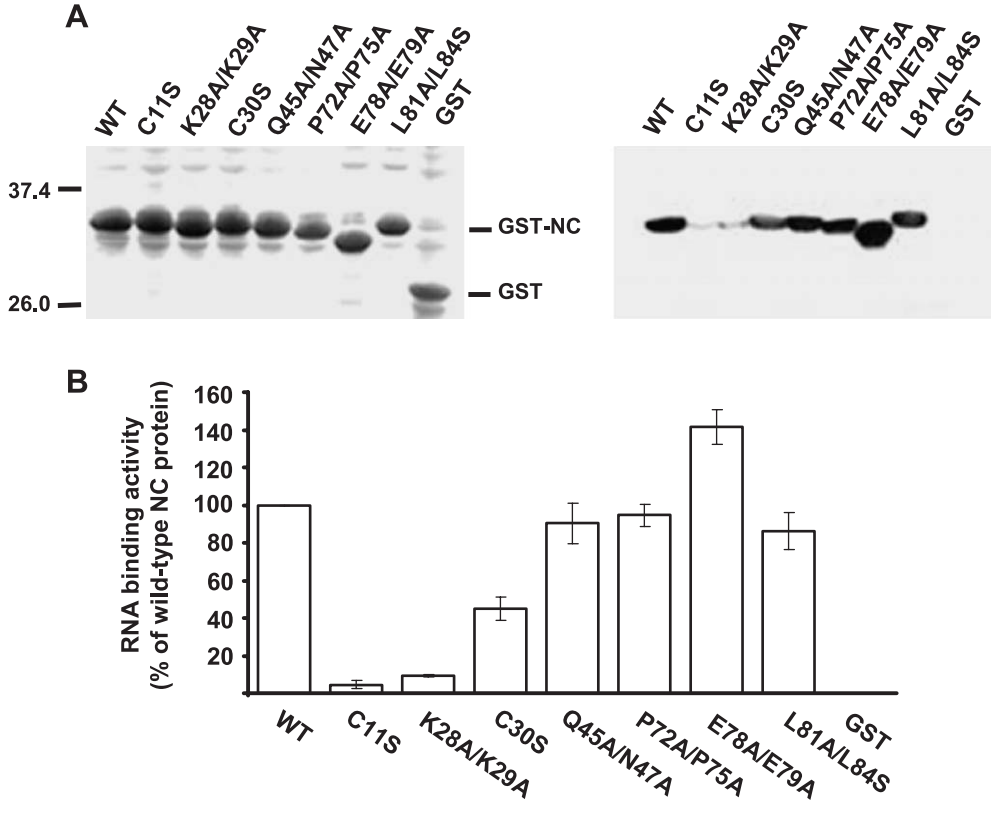

Fig. 3. FIV NC–RNA interactions analyzed in Northwestern blot assays. (A) The wild-type (wt) or mutant FIV NC proteins were expressed as GST fusion proteins, resolved by SDS-gel electrophoresis on 10% polyacrylamide gels, and either stained with Coomassie brilliant blue (left panel) or transferred to nitrocellulose membranes for Northwestern blot analysis (right panel). Membranes were probed with a radioactively labeled riboprobe synthesized from a template corresponding to 5′ FIV sequences (see Materials and methods). The mobilities of the GST and GST fusion proteins are shown, as are the positions of the molecular weight standards. (B) Quantitation of labeled RNA bound to recombinant wild-type (wt) and mutant NC proteins. The RNA binding activity of each NC mutant was referred to that obtained for the wild-type protein (considered as 100%). Data presented are averages of three independent assays ± SD.

mutants bound viral RNA with an efficiency similar to or, in the E78A/E79A mutant, greater than that of the wild-type NC protein. In contrast, the C11S and K28A/K29A mutant NC proteins were highly inefficient at interacting with the viral RNA probe (Fig. 3A). In the case of the C30S NC mutant, viral RNA binding was moderately impaired when compared with that of wild-type FIV NC (Fig. 3A). Quantitation of the amount of labeled RNA bound by the NC proteins in three independent experiments revealed that the Q45A/N47A, P72A/P75A, E78A/E79A, and L81A/L84S mutants bound viral RNA with efficiencies representing 90.5 ± 10.8%, 94.7 ± 5.9%, 141.4 ± 9.3%, and 86.4 ± 9.8%, respectively, of the wild-type NC value (Fig. 3B). In contrast, mutations affecting the proximal zinc finger motif (C11S) or the lysine residues in the region connecting both zinc fingers (K28A/K29A) reduced RNA binding by 90%. Indeed, the C11S and K28A/K29A NC mutants exhibited amounts of bound RNA corresponding to 4.4 ± 2.2% and 9.3 ± 0.6%, respectively, of the wild-type NC value (Fig. 3B). Interestingly, the recombinant NC protein bearing a serine for cysteine substitution in the distal zinc finger motif (C30S mutant) exhibited substantial RNA binding activity representing 45.0 ± 6.1% of that of the wild-type NC protein (Fig. 3B).

It should be mentioned that when we performed Northwestern assays using an RNA probe lacking the R and U5 regions of the FIV 5′ long terminal repeat (LTR), which have been shown to be essential elements of the FIV E signal (Kemler et al., 2002), binding to the FIV NC protein was reduced to 13.3 ± 4.9% of the binding obtained with the full-length probe (average of three assays ± SD). This result underscores the specificity of the FIV NC–RNA interaction detected in our assays.

Effect of mutations at non-zinc-coordinating residues in the FIV NC N-terminal cysteine–histidine motif on viral RNA binding

The results described above indicated that substitution of serine for the first cysteine residue in the proximal zinc finger motif of the FIV NC severely interfered with RNA binding in vitro. The alignment of the amino acid sequences of the FIV, simian immunodeficiency virus (SIV), and HIV-1 NC N-terminal zinc finger motifs reveals that the FIV motif differs at positions 15, 17, and 20 from those of SIV and HIV-1 (Fig. 4A). We therefore decided to investigate whether the amino acids that are specific to the FIV NC proximal zinc finger motif are functionally relevant. To this end, we individually replaced in the first zinc finger motif the amino acids present at FIV NC positions 15, 17, and 20 by those of its SIV counterpart (mutations K15G, P17E, and L20S). The relevance of the conserved proline residue at FIV NC position 17 was further analyzed by introducing the conservative amino acid substitution P17A. In addition, we constructed an FIV NC mutant containing the triple amino acid substitution K15G/P17E/L20S, which exhibits a zinc finger motif virtually identical to that of SIV (Fig. 4A). The NC mutants were expressed as GST fusion proteins and their ability to bind viral RNA was tested in Northwestern assays. Mutants K15G and P17A exhibited an RNA binding activity similar to that of wild-type NC: the amount of RNA probe bound to these mutant proteins was 96.5 ± 18.0% (mutant K15G) and 89.6 ± 8.4% (mutant P17A) relative to that of wild-type NC (Figs. 4B and C). In contrast, the P17E and L20S mutations reduced RNA binding by approximately 60% and 80%, respectively, when compared to the activity displayed by the wild-type NC protein (Figs. 4B and C). Of note, the simultaneous replacement of the FIV NC zinc finger residues at positions 15, 17, and 20 for those present in the SIV NC almost completely blocked the RNA binding ability of the NC protein (Figs. 4B and C). These results indicate that an FIV NC protein containing an SIV-like zinc finger motif at the proximal position is highly inefficient at interacting with FIV genomic RNA in vitro.

Replication of assembly-competent NC mutants in a feline T-cell line

Our experiments to this point indicated that the C30S, Q45A/N47A, and E78A/E79A NC mutants assembled into virions with an efficiency similar to that of wild-type FIV. Moreover, the Q45A/N47A and E78A/E79A NC mutants exhibited an essentially wild-type phenotype with respect to in vitro RNA binding, whereas the C30S mutant displayed substantial RNA binding activity. We therefore examined the ability of the C30S, Q45A/N47A, and E78A/E79A mutant viruses to replicate in a feline T-cell line. MYA-1 cells were infected with the supernatants of CrFK cells transfected with the FIV-14 or the NC mutant proviral DNAs. Virus replication was monitored over time by measuring reverse transcriptase (RT) levels in the cell-free culture supernatants. As shown in Fig. 5, the C30S and Q45A/N47A mutant viruses exhibited replication kinetics similar to that of wild-type FIV. In contrast, the replication of the E78A/E79A mutant in MYA-1 cells was significantly impaired as evidenced by the low levels of RT activity detected over the time period of the experiment. This result indicates that the E78A/E79A NC mutation, which interferes with Gag polyprotein processing, is also detrimental to virus replication in feline T cells.

Discussion

The retroviral NC protein plays several roles during the virus life cycle. As part of the Gag polyprotein, the NC mediates Gag oligomerization, which is critical for particle assembly and directs the packaging of two copies of the viral genomic RNA into virions. In this paper, we identified and characterized molecular determinants in the FIV NC that are

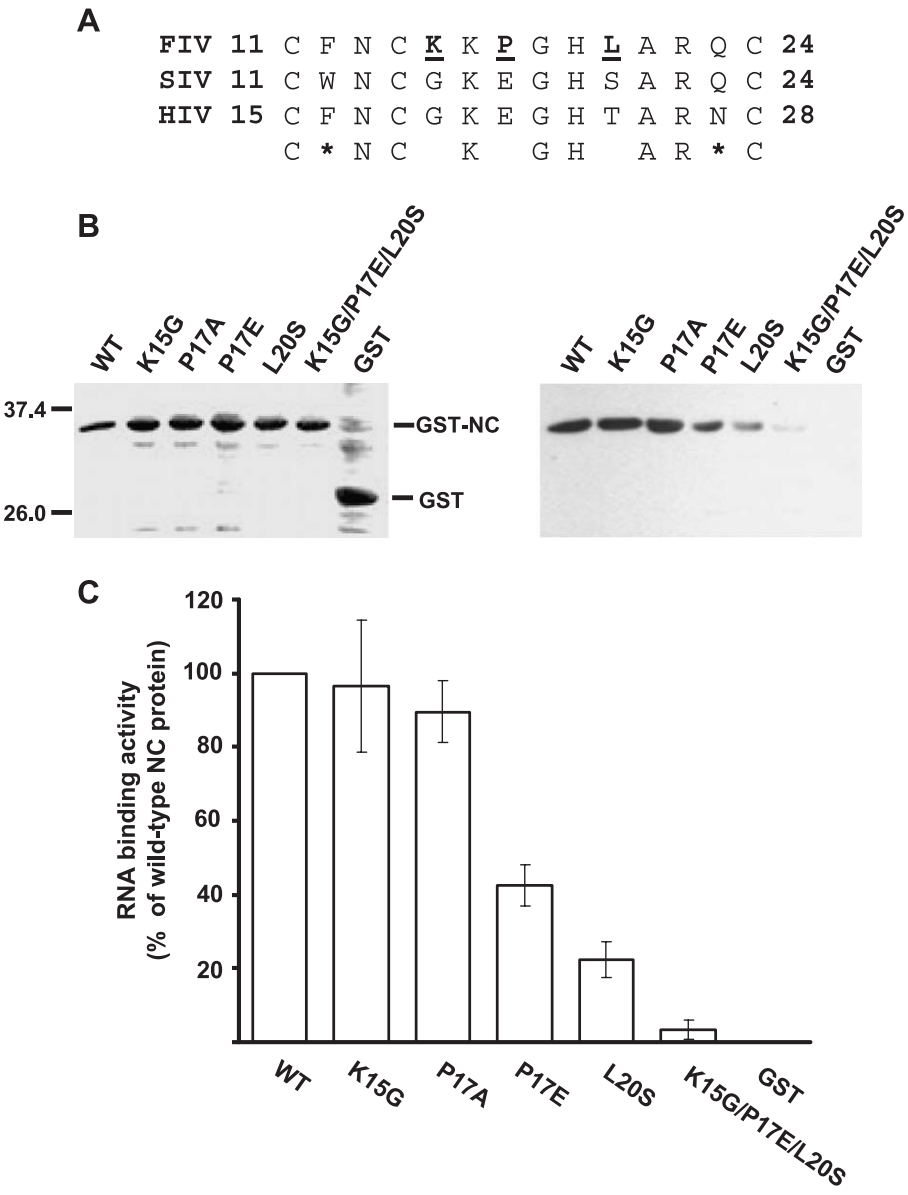

Fig. 4. Effect of mutations at non-zinc-coordinating residues in the FIV NC proximal zinc finger motif on RNA binding activity. (A) Alignment of the amino acid sequence of the proximal zinc finger motif of the FIV (Petaluma), SIV (PBj1.9), and HIV-1 (HXB2) NC proteins. Identical amino acids present in the three sequences are indicated below the alignment, whereas similar amino acids are denoted by an asterisk. The FIV NC residues that were targeted for mutagenesis are shown in boldface letters. (B) Northwestern blot assays of the recombinant FIV NC mutants. The wild-type (wt) or mutant FIV NC proteins were expressed as GST fusion proteins, resolved by SDS-gel electrophoresis on 10% polyacrylamide gels, and either visualized by Coomassie blue staining (left panel) or transferred to nitrocellulose membranes for Northwestern blot analysis as described in Fig. 3 (right panel). (C) Quantitation of labeled RNA bound to recombinant wild-type (wt) and mutant NC proteins. The RNA binding activity of each NC mutant was referred to that obtained for the wild-type protein (considered as 100%). Data presented are averages of three independent assays ± SD.

essential for the biological functions of this protein. Northwestern assays using recombinant FIV NC proteins revealed that replacement of the first cysteine residue in the proximal zinc finger motif by serine (mutation C11S) as well as the K28A/K29A double amino acid substitution that affects the basic region linking the two Cys–His boxes severely impair viral RNA binding with respect to that of wild-type FIV NC. Moreover, amino acid substitutions affecting non-zinc-coordinating residues in the first Cys–His motif, such as the L20S and K15G/P17E/L20S mutations, were found to be detrimental to viral RNA binding. In contrast, the C30S NC mutant, in which the first cysteine residue of the distal zinc finger motif was substituted by serine, exhibited substantial RNA binding activity when compared to that of the wild-type NC protein. Taken together, these results indicate that the proximal zinc finger motif of the FIV NC plays a more prominent role in viral RNA binding than that in the C-terminal position. In HIV-1, the NC proximal Cys–His box is also more sensitive to alteration with respect to genomic RNA packaging than the distal box. Indeed, when two cysteines of

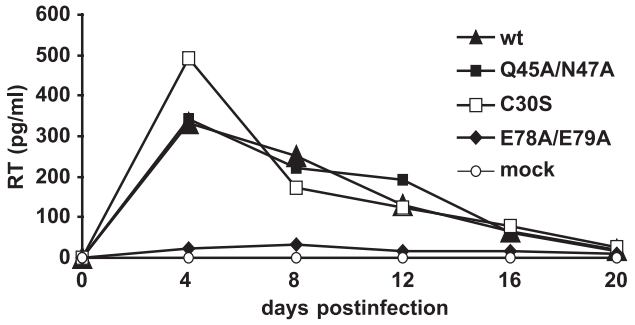

Fig. 5. Replication kinetics of the C30S, Q45A/N47A, and E78A/E79A NC mutant viruses in MYA-1 cells. Virus stocks, obtained by transfection of CrFK cells, were normalized for RT activity and used to infect the MYA-1 cell line. Virus replication was assessed by measuring RT activity at 4-day intervals postinfection. Mock, mock-infected cells.

the first zinc finger motif were changed to tyrosines, encapsidation of genomic viral RNA was drastically reduced (Schwartz et al., 1997). In contrast, when the same mutations were introduced into the second zinc finger motif, the encapsidation efficiency was only slightly affected (Schwartz et al., 1997). Moreover, it has been reported that an HIV-1 NC mutant carrying two versions of the first Cys–His box encapsidated genomic RNA with an efficiency similar to that of wild-type virions, whereas mutants that contained either two copies of the second zinc finger motif or the second motif at the first position were highly inefficient at packaging genomic RNA (Gorelick et al., 1993). Therefore, the relative contribution of the two NC Cys–His boxes to viral RNA binding appears to be similar in both FIV and HIV-1. This situation contrasts with that described for SIV in which the two zinc finger motifs of the NC protein are equally sensitive to mutations with respect to genomic RNA encapsidation (Akahata et al., 2003).

Genetic analysis of the FIV NC protein in the viral context led to the identification of assembly-defective mutants which can be arranged into two groups with respect to their viral RNA binding ability: the C11S and K28A/K29A mutants are highly inefficient at binding RNA and producing particles, whereas the P72A/P75A and L81A/L84S mutants are severely impaired in particle production yet show a viral RNA binding capacity similar to that of wild-type NC. Mutations P72A/P75A and L81A/L84S target highly conserved residues in the C-terminal FIV Gag peptide that results from cleavage at Met66 in the NC domain. In particular, the P72A/P75A mutation was designed to disrupt the PSAP motif in FIV Gag which is also found as P(S/T)AP in the p6 domain of HIV-1 Gag. It has been shown that the P(S/T)AP motif is a Gag "late domain" that recruits the cellular protein TSG101 to exploit the multivesicular body sorting machinery for virus budding (Demirov et al., 2002; Freed, 2002; Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001). The presence of a conserved PSAP motif at the FIV Gag C-terminus whose mutation abrogates virion production suggests that the small C-terminal FIV Gag peptide is functionally equivalent to HIV-1 p6. In this regard, we showed here that disruption of the highly conserved LLDL motif (mutation L81A/L84S) in the FIV Gag C-terminus is also detrimental to virion production, which provides further evidence for the importance of the C-terminal FIV Gag peptide in virion assembly, release, or both. Further studies will be necessary to determine whether the FIV Gag C-terminus is actively recruiting host cellular factors to promote virus budding.

As mentioned above, the impairment in viral RNA binding exhibited by the C11S and K28A/K29A NC mutants is accompanied by a defect in particle production. In this regard, evidence has been presented supporting the concept that NC–RNA interaction plays a central role in retroviral assembly (Johnson et al., 2002; Muriaux et al., 2001; Zhang et al., 1998), retroviral particle stability (Wang et al., 2004), or both. In HIV-1, mutations affecting the basic region linking the NC zinc finger motifs have been shown to interfere with virion production (Cimarelli et al., 2000; Dawson and Yu, 1998), which is in line with the assembly-defective phenotype described here for the K28A/K29A FIV NC mutant. In contrast, the drastic decrease in virion assembly caused by the C11S mutation in the first FIV NC Cys–His box clearly differs from the assembly-competent phenotype exhibited by HIV-1 NC mutants bearing changes at the zinc-coordinating residues in the proximal Cys–His box (Aldovini and Young, 1990; Dorfman et al., 1993; Schwartz et al., 1997). Of note, both HIV-1 NC zinc fingers need to be simultaneously disrupted to inhibit particle assembly (Dorfman et al., 1993). Given that the C11S mutation is likely to interfere only with the zinc-coordinating activity of the FIV NC proximal Cys–His box and, thus, with its ability to interact with the viral RNA, the assembly-defective phenotype observed for the C11S mutant suggests that in FIV, the assembly process is remarkably sensitive to reductions in the genomic RNA binding capacity of the NC domain. That the FIV NC exhibits certain properties different from those of its HIV-1 counterpart is also suggested by the recent data of Moscardini et al. (2002) who have shown that the full nucleic acid chaperone activity of the FIV NC appears to be more dependent on the zinc fingers than that of HIV-1. Further studies will be needed to fully establish the functional relationship between the FIV NC and those of primate lentiviruses.

Materials and methods

Cell lines

CrFK cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (FBS). The feline T lymphoblastoid MYA-1 cell line was maintained in RPMI medium (GIBCO) supplemented with 10% FBS, 100 U/ml interleukin-2 (Roche), and 50 μM β-mercaptoethanol.

Construction of FIV mutant proviruses

All NC mutant proviruses were derived from the infectious molecular clone FIV-14 of the Petaluma isolate (Olmsted et al., 1989). Mutagenesis of the NC-coding region was performed on a *Tth*111I–*Nco*I fragment (nucleotides 924–2499) using the asymmetric PCR-based site-directed mutagenesis method that we have previously described (González et al., 1993; Manrique et al., 2001) using the Elongase enzyme high-fidelity PCR mix (Invitrogen). The *Tth*111I–*Nco*I fragments carrying the desired mutations were substituted for the wild-type counterpart in the parental FIV construct. The presence of the desired mutations in the NC domain was confirmed by DNA sequencing.

Transfections and viral protein analysis

CrFK cells (grown in 60-mm-diameter plates) were transfected with 10 μg of wild-type or mutant FIV proviral DNAs using LipofectAMINE 2000 (Invitrogen). Forty-eight hours post-transfection, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed at 4 °C in lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin] followed by 2-min centrifugation at 16 000 × *g* to remove cellular debris. The postnuclear supernatants were analyzed by Western blotting (see below). To purify virions, the cell culture supernatants were filtered through 0.45-μm-pore-size syringe filters, and virions were pelleted from the clarified supernatants by ultracentrifugation (100 000 × *g*, 90 min, 4 °C) through a 20% (w/v) sucrose cushion (Manrique et al., 2001). Cell- and virion-associated proteins were resolved on SDS-10% polyacrylamide gels, blotted onto nitrocellulose membranes and analyzed by Western blotting coupled with an enhanced chemiluminescence assay (ECL, Amersham Biosciences) as previously described (Manrique et al., 2001). FIV Gag-related proteins were detected by using an anti-FIV CA monoclonal antibody (PAK3-2C1) obtained through the NIH AIDS Research and Reference Reagent Program.

Cloning and expression of recombinant NC proteins

The coding regions for the wild-type or mutant NC proteins were PCR amplified from the wild-type or mutant proviral DNAs using two primers that introduce *Bam*HI and *Sma*I restriction sites at the 5′ and 3′ ends of the DNA product, respectively. The amplified and *Bam*HI/*Sma*I-digested fragments were cloned into the corresponding sites of the pGEX-2T plasmid vector (Amersham Biosciences) which allowed the expression of the NC genes as fusions with *Schistosoma japonicum* GST. Overnight cultures of *E. coli* DH5α strain transformed with pGEX-2T or recombinant plasmids were diluted 1:25 with fresh medium and grown for 3 h at 37 °C before inducing with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After a further 3- to 5-h incubation at 37 °C, the cells were pelleted and resuspended in ice-cold PBS. Cells were lysed by sonication followed by centrifugation at 16 000 × *g* during 10 min at 4 °C. The supernatant was collected and the GST or GST fusion proteins were purified by affinity chromatography using glutathione Sepharose 4B according to the manufacturer's instructions (Amersham Biosciences). The concentration of the purified proteins was determined by the method of Bradford.

In vitro synthesis and labeling of FIV RNA

For in vitro transcription of the FIV RNA containing the packaging signal (Kemler et al., 2002), the DNA fragment corresponding to nucleotides 216–947 of the FIV proviral DNA was PCR amplified and cloned into the *Apa*I and *Sal*I sites of the pGEM-5Zf plasmid (Promega). The cloned fragment corresponds to the R and U5 regions of the FIV 5′ LTR together with the first 320 nucleotides of the *gag* gene. The resulting plasmid was linearized with *Sal*I and used as template for RNA synthesis. Radiolabeled RNA was synthesized with 20 U of T7 RNA polymerase (Promega) in a final volume of 20 μl containing 1 μg of linear plasmid; 40 mM Tris–HCl (pH 7.9); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 10 mM DTT; 40 U RNasin ribonuclease inhibitor (Promega); 0.5 mM (each) ATP, GTP, CTP; 12.5 μM UTP; and 100 μCi [α-^{35}S]UTP (1250 Ci/mmol, NEN). The reaction mixture was incubated 1 h at 37 °C followed by treatment with 1 U of RNAse-free DNase I (Promega) and further incubated at 37 °C for 15 min to remove the DNA template. The reaction was extracted twice with a 1:1 mixture of phenol and chloroform and then precipitated twice in ethanol. The RNA product was resuspended in water and stored at −80 °C. An RNA probe lacking the R and U5 regions of the FIV LTR was generated by in vitro transcription of a pGEM-5Zf plasmid containing the FIV-14 region spanning nucleotides 457–904. This probe includes the last 171 nucleotides of the 5′ untranslated region of the FIV genome and the first 177 nucleotides of the *gag* gene.

Northwestern blot

The GST or GST-NC proteins were separated by SDS-10% polyacrylamide gels and electroblotted onto nitrocellulose membranes. The blots were incubated 16 h at 4 °C in Northwestern (NW) buffer [30 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.5), 400 mM KCl, 10 μM $ZnCl_2$, 2 mM DTT, and 500 μg/ml heparin]. Membranes were incubated at room temperature for 2 h in NW buffer containing 3 to 6 × 10^6 cpm of radioactively labeled RNA and then washed twice at room temperature for 10 min in NW buffer containing heparin and twice in buffer lacking heparin. The blots were dried and subjected to autoradiography. Quantitation of the amount of RNA probe bound to the blots was performed by Phosphor-

Imager analysis using the ImageQuant software (Molecular Dynamics).

RT assays

Quantitation of virion-associated RT in cell-free culture supernatants from transfected or infected cells was performed by using a nonisotopic assay kit (Roche Diagnostics) as we have previously described (Manrique et al., 2003). RT levels were calculated relative to the recombinant HIV-1 RT enzyme provided in the kit.

Virus replication in MYA-1 cells

To obtain replication kinetics data in the feline T-cell line MYA-1, virus stocks were first generated by transfection of CrFK cells as described above. Equal RT activity in volume-adjusted supernatants was added to 2×10^5 MYA-1 cells and allowed to adsorb for 4 h. The cells were washed twice with PBS to remove residual virus and incubated with fresh medium. Cell cultures were split at a 1:2 ratio every 4 days with fresh medium and aliquots of culture supernatants were frozen at −80 °C for RT determination at the conclusion of the experiment.

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