Molecular and functional interactions of cat APOBEC3 and feline foamy and immunodeficiency virus proteins: Different ways to counteract host-encoded restriction

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A B S T R A C T
Defined host-encoded feline APOBEC3 (feA3) cytidine deaminases efficiently restrict the replication and spread of exogenous retroviruses like Feline Immunodeficiency Virus (FIV) and Feline Foamy Virus (FFV) which developed different feA3 counter-acting strategies. Here we characterize the molecular interaction of FFV proteins with the diverse feA3 proteins. The FFV accessory protein Bet is the virus-encoded defense factor which is shown here to bind all feA3 proteins independent of whether they restrict FFV, a feature shared with FIV Vif that induces degradation of all feA3s including those that do not inactivate FIV. In contrast, only some feA3 proteins bind to FFV Gag, a pattern that in part reflects the restriction pattern detected. Additionally, one-domain feA3 proteins can homo- and hetero-dimerize in vitro, but a trans-dominant phenotype of any of the low-activity feA3 forms on FFV restriction by one of the highly-active feA3Z2 proteins was not detectable.

Identifying viral factors that shaped the cellular repertoire of A3 proteins and their evolution requires a comprehensive understanding of the viral factors targeted by the restriction system and those developed by the viruses to circumvent it. Recent studies on Feline Immunodeficiency Virus (FIV) presented evidence that the viral targets and counter-actions are highly related to that of Human Immunodeficiency Virus (Stern et al., 2010; Wang et al., 2011; Zielonka et al., 2010). However, little is known about other feline retroviruses that likely played also an important evolutionary role for the Felidae (Löchelt et al., 2005; Münk et al., 2008). In addition, this active co-evolution also leads to the establishment of cross-species transmission barriers since the viral counter-defense is in general ineffective or only partially active against heterologous restriction factors of non-authentic host species (Baumann, 2006; Münk et al., 2010; Zielonka et al., 2010).

Spumaretro- or foamy viruses (FVs) are a distinct group of retroviruses that are promising candidates for the development of novel viral vectors for gene delivery and vaccination (Rethwilm, 2007; Schwantes et al., 2003; Trobridge, 2009). Similar to the much better studied lentiviruses, FVs have a complex genetic makeup consisting of the canonical retroviral gag, pol, and env genes and the regulatory bel 1/tas gene encoding the viral transactivator (Lia, 1999; Rethwilm, 2010). In addition, the accessory bet gene that is generated via splicing counteracts cellular A3-mediated restriction and may have a role in particle release and in establishing viral persistence (Alke et al., 2001; Löchelt et al., 2005; Russell et al., 2005; Saib et al., 1995). In contrast to the unique

Introduction

The replication of exogenous pathogens is often inhibited by specific cellular defense mechanisms, e.g. in the form of pathogen-specific restriction factors (Bieniasz, 2004). APOBEC3 (A3) cytidine deaminases have recently been shown to efficiently restrict the replication and spread of exogenous and endogenous retroviruses, retroid elements and other, distantly related viruses (Harris and Liddament, 2004). In face of this cellular restriction, retroviruses developed different, often highly specific strategies to counteract this restriction (Chiu and Greene, 2008; Cullen, 2006; Münk et al., 2010). The ongoing struggle of cellular defense and viral counter-action results in a dynamic co-evolution of the partners involved (LaRue et al., 2009). In cats, this resulted in a complex A3 locus that underwent a dramatic recent expansion resulting in three related feA3Z2 genes (feA3Z2a, -b, and -c) as well as one feA3Z3 gene, all encoding one-domain A3 proteins (Münk et al., 2008). In addition, a two-domain feA322-Z3 cytidine deaminase is expressed by complex read-through transcription (Münk et al., 2008; Zielonka et al., 2010).
molecular biology of FVs, insights into FV replication in the infected individual, the sites of FV replication, the authentic target cell(s), the potential disease association and the extent of virus replication during lifelong persistence are limited at present (Bastone et al., 2003; Linial, 2000; Saib, 2003). In this context, our studies on Feline Foamy Virus (FFV) replication in vitro but especially in cats with respect to host-virus interaction and interaction with other pathogens aim at enhancing understanding of these aspects of FV biology (Alke et al., 2000; Romen et al., 2006; Schwantes et al., 2003).

Different FVs of non-human primates have the capacity to cross species barriers to other primates and pose even a significant risk of zoonotic, inter-species transmission to humans (Heneine et al., 2003; Switzer et al., 2004). For instance, the prototypic/human FV isolate PFV has been shown to be the end-product of a zoonotic transfer of a chimpanzee FV to humans (Herchenröder et al., 1995). No disease has been associated with the limited number of well-documented zoonotic transmissions of FVs to humans (Khan, 2009). The apparent ease of interspecies transmissions of primate FV among primates implies that the active and extensive co-evolution of host-encoded restriction factors and the viral counter-defense have been insufficient to establish stable barriers towards cross-species transmission (Leendertz et al., 2008, 2010). On the other hand, the control and containment of FV replication by different restriction systems may explain the apparent pathogenicity of FVs in their cognate as well as in their heterologous hosts (Bastone et al., 2003; Münk et al., 2010; Saib, 2003).

By comparing the APOBEC3-mediated restriction of FFV and FIV and the viral counter-defense via FFV Bet and FIV Vif, we showed that there are clear species-specific differences in APOBEC3-mediated restriction (Münk et al., 2008). Importantly, we show here that both FIV Vif and FFV Bet inactivate all known feline APOBEC3 proteins independent of whether they do or do not restrict the respective virus. Furthermore, Bet and Vif function by fundamentally different means: FIV Vif (like primate lentiviral Vif) leads to proteasomal degradation of all feA3 proteins while FFV Bet strongly binds all feA3s without decreasing their stability. This indicates that a Bet-mediated sequestration, masking, or the retention of feA3 proteins results in their functional inactivation. Finally, feA3 proteins bind to FFV Gag independent of whether they efficiently restrict FV replication.

Results

Different A3 cytidine deaminases have been recently identified in the cat genome and their overall pattern of anti-retroviral activity against feline lenti-, onco-, and spumaviruses was defined: feA3Z2 and –Z2b-Z3 have only minor effects on FFV replication, the feA3Z2b and -c isoforms are strongly suppressive while feA3Z2a is less active (Münk et al., 2008). It was also shown that FFV Bet very efficiently counteracts feA3Z2b-mediated restriction by a unique mechanism independent of decreasing the stability of the restriction factor (Lochelt et al., 2005; Münk et al., 2008). Here we thoroughly determine the functional and physical interaction of the feA3s with proteins of FFV, the feline retrovirus that is strongly affected by these cellular restriction factors. In addition, we study functional and physical interactions of the different feA3 proteins.

Interaction of feA3 proteins and FFV Bet

We recently showed that FFV Bet specifically interacts with feA3Z2b, the feA3 form that is most potent in restricting FFV (Lochelt et al., 2005). We thus studied here whether Bet also binds the other feA3Z2 variants and/or those feA3 proteins that are not active against FFV (Münk et al., 2008). For this purpose, 293 T cells were co-transfected with either wt FFV clone pCF-7 or pCF-BetMCS, an FFV derivative encoding a full-length but non-functional Bet (Alke et al., 2001) and with plasmids encoding HA-tagged feA3Z2a, -b, -c forms (Fig. 1A) or feA3Z2b, -Z3, and –Z2b-Z3 (Fig. 1B). Cell lysates harvested 48 h post transfection (p.t.) were subjected to co-immuno-precipitation (co-IP) using an HA tag-specific monoclonal antibody. Subsequently, precipitated proteins were detected by immuno-blotting. While wt FFV Bet was efficiently co-precipitated by all three feA3Z2 iso-forms (Fig. 1A), the mutant BetMCS carrying heterologous amino acid sequences in a protein motif directly downstream of the Bel1/Tas coding sequence (Alke et al., 2001) was not precipitated at all (top panel) although feA3Z2a to -c expression levels were comparable (Fig. 1A, third panel from top). The result was not due to different steady-state

![Fig. 1. The known feA3 proteins interact with wt but not with mutant FFV Bet. A. For co-IP experiments, 293 T cells were co-transfected with HA-tagged feA3Z2a, -b and -c expression plasmids and pcDNA3.1 control DNA together with wt pCF-7 and bet mutant pCF-BetMCSs. Cells were harvested 2 d p.t. and extracts used for co-IP with HA-specific antibodies. Co-precipitated proteins were detected by immuno-blotting. As shown in the top panel, only wt but not mutant Bet-MCS was co-precipitated. Below, immuno-assays of cell extracts document proper protein expression and identical loading. B. Similarly, HA-tagged feA3Z2b, -Z3 and -Z2b-Z3 expression plasmids and pcDNA3.1 control DNA together with wt pCF-7 and bet mutant pCF-BetMCSs genomes were co-transfected into 293 T cells. Cell extracts were used for co-IP assays using HA-specific antibodies. As shown in the top two panels, only wt but not MCS-mutant Bet was co-precipitated with all different feA3 forms (the one-domain feA3Z2b and -Z3 forms marked by an arrow co-migrate with the IgG light chains used for co-IP) using an HA tag-specific monoclonal antibody. Subsequently, precipitated proteins were detected by immuno-blotting. While wt FFV Bet was efficiently co-precipitated by all three feA3Z2 iso-forms (Fig. 1A), the mutant BetMCS carrying heterologous amino acid sequences in a protein motif directly downstream of the Bel1/Tas coding sequence (Alke et al., 2001) was not precipitated at all (top panel) although feA3Z2a to -c expression levels were comparable (Fig. 1A, third panel from top). The result was not due to different steady-state
levels of Bet (second panel from top) or loading differences (Fig. 1A, bottom panel). In addition, precipitation with a normal mouse serum instead of the anti-HA serum did not result in precipitation of either feA3 or Bet proteins (data not shown). Correspondingly, we analyzed whether feA3Z2 and -Z2b-Z3 similarly interact with Bet (Fig. 1B). Similar to feA3Z2b used as positive control, feA3Z2 and -Z2b-Z3 exclusively interacted with wt but not with mutated Bet (top panel). Precipitation of feA3 proteins as well as Bet protein levels and overall loading were comparable between the different samples (Fig. 1B).

The interaction of Bet with the known feA3 proteins is independent of additional FFV proteins as detected when an FFV Bet-expression plasmid devoid of any other FFV proteins was co-expressed with the different feA3s (data not shown).

**FFV Bet does not induce proteolytic degradation of feA3 proteins**

We next compared the steady state protein levels of HA epitope-tagged feA3 proteins in the presence of either FFV Bet or FIV Vif and compared them to those without any of these viral A3 defense factors. A codon-optimized and V5-tagged FIV vif that yields enhanced Vif protein levels (Münk et al., 2008) was used. 293 T cells were harvested 2 d.p.t. and whole cell lysates were analyzed for the presence of the different feA3 proteins (Fig. 2, left-hand panel, anti HA antibody). While steady-state levels of feA3Z2b, -Z3 and -Z2b-Z3 were not affected by Bet, the same proteins were undetectable in cells co-expressing VIF Vif. Comparable expression of Bet as well as Vif was confirmed by immuno-blotting. These findings confirm previous data that FIV Vif induces degradation of all feA3 proteins independent of whether they do (feA3Z2b-Z3 and -Z3) or do not (feA2Z2) suppress FIV replication (Münk et al., 2008; Stern et al., 2010; Wang et al., 2011; Zielonka et al., 2010). To confirm that FIV Vif induced proteasomal degradation of feA3 proteins, the above experiment was in parallel performed in the presence of N-acetyl-Leu-Leu-norleucinal (ALLN), an efficient inhibitor of cysteine proteases and the proteasome (Fig. 2, right-hand panel). As expected, ALLN had no or only minor effects on feA3 stability in the presence or absence of Bet, while ALLN at least partially restored feA3Z2b, -Z3 and -Z2b-Z3 when FIV Vif was co-expressed. Parallel studies using MG132 which more specifically inhibits proteasomal functions confirmed the proteasome-mediated degradation of feA3 proteins via FIV Vif but not via FFV Bet (data not shown).

The one-domain feA3Z2 and -Z3 proteins homo- and hetero-multimerize

The single-domain human A3 (huA3) proteins dimerize (Bennett et al., 2008; Shindo et al., 2003; Shlyakhtenko et al., 2011; Stauch et al., 2009) and even the two-domain huA3G may have to multimerize in order to restrict HIV (Huthoff et al., 2009). Importantly, it had been recently suggested that FFV Bet can interfere with huA3C dimerization (Perkovic et al., 2009). To determine whether feA3 proteins homo- and hetero-multimerize, protein pull-down experiments were performed. Glutathione-S-transferase (GST)-tagged feA3Z2b and -Z3 fusion proteins were expressed and purified from recombinant bacteria. The feA3 fusion proteins and a bovine FV (BFV) GST-Env protein used as a control were incubated with extracts from 293 T cell expressing feA3Z2b, -Z3, and -Z2b-Z3 or transfected with an empty vector. Bound proteins were precipitated with the GST–glutathione interaction and detected by immuno-blotting (Fig. 3). Pull-down experiments in the absence of additional FFV proteins showed specific homo- and heterodimeric interaction of the one-domain feA3Z2b and -Z3 proteins whereas the two-domain feA3Z2b-Z3 did not bind any of the one-domain feA3 proteins. The specificity of these interactions was confirmed by the negative controls consisting of feA3-deficient cell extract or the heterologous GST-tagged BFV Env ectodomain (Romen et al., 2007) and the positive control showing the feA3–FFV Gag interactions (see below).
with an HA-specific antibody (right-hand margin). The lysates were incubated with affinity-purified BFV GST-Env (negative control), feA3Z2b, -Z3 and -FFV Gag fusion proteins (as indicated below the blot). Complexes were harvested by affinity interaction with glutathione-coated beads. HA-tagged feA3 proteins in the pull-down fractions were detected by immuno-blotting with an HA-specific serum.

No trans-dominant negative effect of any of the feA3 proteins in co-expression studies

Since the one-domain feA3 proteins show different capacities to suppress FFV infectivity (Münk et al., 2008) and since they may have to multimerize to gain enzymatic activity (Bennett et al., 2008; Shindo et al., 2011), we analyzed whether co-transfection of cells with identical amounts of feA3Z2b together with the other one-domain feA3 forms and the Bet-deficient FFV genome pCF-BBtr results in altered levels of FFV restriction. The activity of the strongly restricting feA3Z2-family member feA3Z2b was not or only minimally affected by expression of the other feA3 proteins (Fig. 4). The data from three independent assays indicate that there is neither synergism nor antagonism of the one-domain feA3 forms and the Bet-deleted FFV-vectors are restricted by feAZ2b and Gag. The feA3Z2-family member feA3Z2a also showed a strong and RNA-independent interaction with FFV-Gag, whereas the weak association of feAZ2c with Gag was only detectable without RNaseA (Fig. 5B and data not shown).

Interaction of feA3 forms with FFV-Gag

To investigate whether all feA3 proteins interact with FFV-Gag and to find out whether the interaction is RNA-dependent, protein pull-down analyses were performed. For this purpose, the full-length FFV gag was expressed as GST-Gag fusion protein in bacteria and purified by affinity chromatography using glutathione-coupled agarose beads. The GST-tagged FFV Gag was mixed with 293 T cell lysates containing or lacking the different HA-tagged feA3 proteins. To determine the influence of RNA on the feA3-Gag interactions, pull-down assays were performed in the presence or absence of 50 μg/ml RNaseA. Proteins bound to FFV Gag were precipitated via the GST-glutathione interaction and detected by immuno-blotting using HA tag-specific antibodies. Consistent with previous data (Lochelt et al., 2005), feA3Z2b bound Gag while feA3Z2b-Z3 displayed an even stronger affinity to GST-tagged FFV Gag (Fig. 5A, see also Fig. 3). In contrast, only low-level interaction of feA3Z3 with FFV-Gag was detectable and there was no unspecific interaction with the GST moiety. The interactions of feA3Z2b or -Z2b-Z3 with FFV Gag were only slightly affected by RNaseA treatment but RNA seemed to stabilize the weak interaction of feAZ3 and Gag. The feAZ2-family member feAZ2a also showed a strong and RNA-independent interaction with FFV-Gag, whereas the weak association of feAZ2c with Gag was only detectable without RNaseA (Fig. 5B and data not shown).

Co-localization of feAZ2b and FFV Gag

To confirm the Gag-feA3 interaction shown above by another technique, co-localization studies by confocal microscopy were performed as recently described (Liu et al., 2011). In these studies, FFV Gag was detected with a capsid-specific serum (red, left-hand column), feA3Z2b-HA was detected by an HA-specific antibody (green, central column) and nuclei were stained with Hoechst 33342 (blue). Plasmids encoding wt FFV Gag with a mostly peri-nuclear and homogeneous or fine-granular cytoplasmic localization (Fig. 6, second row) and a membrane targeted myr-Gag with a typical cytoplasmic speckled, membrane, and/or vacuolar localization (Fig. 6, fourth row, Liu et al., 2011) were used to transfet HeLa cells together with a plasmid encoding feA3Z2b (as indicated at the right side of the panels). In the absence of Gag, feA3Z2b is homogenously distributed close to the nucleus (top panel), while co-expression with both Gag proteins resulted in a fine- or coarse-granular localization depending on whether it interacted with FFV wt or myr-Gag (middle and bottom rows). The merge of the Gag and feA3Z2b staining is shown in the right-hand column as a clear orange to yellow co-localization of both proteins. The re-distribution of feA3Z2b by co-expressed wt or myr-Gag clearly confirms molecular interactions of both proteins.

Replication-deficient, Bet-deleted FFV vectors are restricted by feAZ2b

To determine whether replication-deficient, bet-deleted FFV-vectors are also restricted by feA3Z2b, the env-bel-deleted β-gal-encoding FFV-vector pCF-ubi-lacZ trans-complemented with FFV Env was analyzed (Bastone et al., 2007). 293 T cells were co-transfected with the vector constructs and either an empty control plasmids or the FFV Bet plasmid pBC-Bet and with pfeA3Z2b encoding the most active restriction factor of FFV as indicated (Fig. 7). Vector titers were determined as β-gal transfer to CRFK cells which are highly susceptible towards FFV and expressed as transducing units per ml cell culture supernatant (Bastone et al., 2007).

**Fig. 3.** Dimerization of the one-domain feAZ2b and -Z3 proteins. Protein pull-down assays were performed using cell lysates harvested 2 d.p.c. of 293 T cells with pcDNA3.1 DNA and plasmids encoding HA-tagged feAZ2b, -Z3 and -Z2b-Z3 (as given at the right-hand margin). The lysates were incubated with affinity-purified BFV GST-Env (negative control), feAZ2b, -Z3 and -FFV Gag fusion proteins (as indicated below the blot). Complexes were harvested by affinity interaction with glutathione-coated beads. HA-tagged feAZ3 proteins in the pull-down fractions were detected by immuno-blotting with an HA-specific serum.

**Fig. 4.** Co-expression of different one-domain feAZ3 proteins does not alter restriction of Bet-deficient FFV. 293 T cells were co-transfected with the Bet-deficient pCF-BBtr and 1:1 mixtures of plasmids encoding feAZ2b and the different feAZ3 forms or with the empty pcDNA vector. Two d.p.c., cell culture supernatants were assayed for FFV infectivity using the FeFAB titration assays. Mean titers of three independent experiments are presented as FFU/ml cell culture supernatant, standard deviations are given by error bars.
Co-expression of Bet only slightly decreased vector titers, while feA3Z2b resulted in a very dramatic decrease of the vector titer. Co-expression of Bet partially relieved feA3Z2b restriction of the pCF-ubi-lacZ gene transfer vector. Proper expression of FFV Bet and feA3Z2b was confirmed by immuno-blot analysis of cell extracts (data not shown).

**Discussion**

In this study, previous reports on the antiviral activity of APOBEC3 cytidine deaminases and their inhibition by the FV Bet protein are confirmed and substantially extended (Löchelt et al., 2005; Münk et al., 2008; Russell et al., 2005). In a feline retrovirus model we show here that all known A3 proteins of cats are bound by FFV Bet but that only the Z2 domain-containing feA3 restriction factors efficiently interact with FFV Gag in vitro which may be a prerequisite for their biologically activity (Bennett et al., 2008; Shindo et al., 2003; Shlyakhtenko et al., 2011; Stauch et al., 2009). Since different A3 forms are co-expressed in single cells or organs (Koning, et al., 2009; Münk et al., 2008; Refsland et al., 2010), the presence of inactive or low-activity forms may decrease the activity of highly active A3s. However, corresponding studies described here did not show trans-dominant negative effects of the less active feA3-forms on feA3Z2b-mediated restriction of FFV. In pull-down experiments, formation of homo- and hetero-dimers of the single-domain feA3 proteins was not inhibited by FFV proteins including Bet (data not shown) which contrasts recent co-IP experiments where PFV Bet was shown to prevent human A3C dimerization (Perkovic et al., 2009).

While the pattern of restriction of the different exogenous feline retroviruses by the feA3 proteins had been published recently (Münk et al., 2008), functional and mechanistic studies on their interaction with FFV and a direct comparison to FIV Vif, the phylogenetically unrelated but functionally equivalent counterpart of lentiviruses had not been performed so far. The data presented here confirm that also non-primate lentivirus Vif induces proteasomal degradation of host A3 proteins in contrast to the FV Bet proteins that do not affect steady state levels of evolved than that of other placental mammals, for instance mice and dogs (LaRue et al., 2009; Münk et al., 2008). In analogy to other A3 proteins and as shown in this study, the feline one-domain A3Z2 and -Z3 proteins homo- and hetero-dimerize which is presumably a prerequisite for their biologically activity (Bennett et al., 2008; Shindo et al., 2003; Shlyakhtenko et al., 2011; Stauch et al., 2009).

The feA3 locus and thus the repertoire of feA3 protein is less complex than that of humans, monkeys or horses but significantly more...
A3 restriction factors (Löchelt et al., 2005; Perkovic et al., 2009; Russell et al., 2005; Stern et al., 2010; Wang et al., 2011; Zielonka et al., 2010). Thus, induction of proteasomal degradation of A3 proteins is the conserved mechanism of lentivirus Vif proteins while the FV Bet proteins studied so far bind a very broad range of A3 proteins of their host species which likely leads to A3 inactivation, for instance by interfering with A3 dimerization and/or particle incorporation (Löchelt et al., 2005; Perkovic et al., 2009). Similarly, FIV Vif induces degradation of all major feA3 forms independent of whether they restrict FIV replication or not. This feature of FIV Vif is in contrast to the situation in HIV-1 where Vif clearly inactivates human A3DE, -F and -G but does not target A3A and A3B while susceptibility of the other human A3 forms is controversial (summarized in Albin and Harris, 2010). The biological significance of the breadth of protection by FIV Vif but also the breadth of feA3 binding by FFV Bet is currently not understood but may be studied in transgenic cats similar to those that have been recently established (Wongsrikeao et al., 2011). The broad protection against restriction may reflect a much longer host-virus co-evolution of cats with both feline retroviruses versus the situation in human where HIV was only recently acquired by men while repeated zoonotic transmissions of simian FVs to human beings have not resulted in the stable establishment of FVs in the human population (Heneine et al., 2003; Pecon-Slattery et al., 2008; Sharp and Hahn, 2008; Switzer et al., 2005).

Fig. 6. Co-localization of feA3Z2b-HA with wt and myristoylated Gag proteins. HeLa cells were transfected with a plasmid encoding HA-tagged feA3Z2b-HA and/or FFV wt and myr-Gag expression plasmids as indicated on the right side of the image. For the detection of FFV Gag, a capsid-specific serum was used (red, left-hand column), feA3Z2b-HA was detected by an HA-specific antibody (green, central column) and nuclei were stained with Hoechst 33342 (blue). The merge of the Gag and feA3 staining is shown in the right-hand column. In transfected HeLa cells, feA3Z2b-HA (green) is distributed predominantly in the peri-nuclear region. In the co-transfected cells, feA3 is changing its localization and it is co-localizing with wt/myr-add-Gag (orange to yellow). The inserted bars represent 5 μm.
Ongoing studies aimed at characterizing A3-mediated restriction, this would be indicative of an extremely long co-evolution of FVs as the most ancient retroviruses and host-encoded A3 restriction (Switzer et al., 2005). Thus, studying the molecular mechanisms of anti-viral restriction and viral counter-defense should shed new insights into the evolution of this system and its past and current contribution to virus replication capacity and virulence as well as pathogenicity or apathogenicity, resp.

Material and methods

Cell culture and virological methods

293 T cells and FFV FAB cells used for titration of FFV were propagated as described (Zemba et al., 2000). Transfection of sub-confluent 293 T cells grown in 20 and 60 cm² dishes with plasmid DNA using a modified Calcium-Phosphate method was done as recently described (Zemba et al., 2000). The infectivity of wt and bet-deficient FFV in presence of the A3 variants was assayed by the β-galactosidase- assay using FFV-FAB cells grown in 24-well plates (Zemba et al., 2000). In order to inhibit the cellular proteasome, cells were treated from 12 to 40 h p.t. with 25 μM of the proteasome inhibitor ALLN (dissolved in DMSO) supplemented to the medium.

Production of the replication-deficient and bet-deleted β-gal-encoding FFV vector pCF-ubi-lacZ trans-complemented with FFV Env and quantification of marker gene transfer were done as previously described (Bastone et al., 2007).

Plasmids and DNA transfection

FFV wt and Bet mutant plasmids pFFV-BBtr and pFFV-MCS and the eukaryotic FFV Bet expression plasmid have been described previously (Alke et al., 2001; Zemba et al., 2000). In pFFV-BBtr, the 387-residue-wt-Bet is truncated after amino acid 116, whereas in pFFV-MCS few residues are exchanged and inserted at the same site (Alke et al., 2001). To increase gene expression, both Bet mutations were cloned into the CMV-IE promoter-driven FFV pCF-7 (Schwantes et al., 2002), resulting in mutants pCF-BBtr and pCF-MCS. Isolation and cloning of feA3Z2a, -b, -c, feA3Z2b and feA3Z2bZ3 forms previously designated APOBEC3C-a, -b, -c, feAPOBEC3H and feAPOBEC3CH have been described previously (Löchelt et al., 2005; Münk et al., 2007, 2008; Zielonka et al., 2010). In some experiments, a feA3Z3 expression clone lacking un-translated leader sequences and carrying an Asc to Asc exchange of the second feA3Z residue was used. Expression plasmids for FFV wt Gag and myr-Gag (pBC-wtGag and pBC-ScrGag-add, resp.) have been described recently (Liu et al., 2011).

Bacterial expression vectors for Glutathione-S-transferase (GST)-tagged FFV Gag and Bet as well as BFV Env have been described recently (Romen et al., 2006, 2007).

Immuo-precipitation and immuno-blotting

For co-immuno-precipitation (co-IP) of FFV Bet or FFV Bet-MCS and feA3 variants, 293 T cells were transfected with 4ug of feA3Z2a-HA, feA3Z2b-HA, feA3Z2c-HA, feA3Z3-HA and feA3Z2b-Z3-HA expression plasmid and 12ug of pCF-7 or pCF-BetMCS. 2 d p.t., cells were lysed in TLB (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 2 mM EDTA, pH 8, 1% Triton X-100, 50 mM Na β-glycerophosphate and protease inhibitors) and lysates were cleared by centrifugation. To increase specificity, the supernatants were pre-incubated with normal mouse serum (Sigma-Aldrich, Munich) coupled to protein-G-Sepharose (Amersham Bioscience, Uppsala, Sweden) for 60 min, at 4°C. After removing the supernatant the beads were washed 3 times with TLB. The supernatants were incubated with anti-HA-IgG

Fig. 7. FFV-based replication-deficient vectors are restricted by feA3Z2b. 293 T cells were co-transfected with the bet- and replication-deficient FFV vector pCF-ubi-lacZ and an FFV Env expression plasmid and either without any other effector plasmid or together with plasmids encoding FFV Bet, feA3Z2b or both (as indicated). Two d p.t., cell culture supernatants were assayed for lacZ marker gene transfer using CRFK target cells. Titers are presented as FFU/ml cell culture supernatant.
from mouse (Covance, Muenster) coupled to protein-G-sepharose for 60 min. at 4 °C and washed three times with TLE. After boiling in electrophoresis sample buffer, samples of the preincubation and the immuno-precipitation were subjected to SDS-PAGE and immuno-blotting (Alke et al., 2001). Detection of feA322 variants was performed with monoclonal anti-HA-IgG (Covance, Muenster). Detection of FFV-Bet with the FFV Bel2/Bet antisem is has been described previously (Alke et al., 2001). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia, Sigma-Aldrich) and visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia). For immuno-blotting of the lysates, identical amounts of cell extracts were used as determined by Roti-Quant protein quantification (Roti, Karlsruhe, Germany).

**Immunofluorescence and confocal microscopy**

HeLa cells were grown on coverslips and transfected with 1 μg FFV Gag (wt or myr) expression plasmid and 1 μg of pfeA322b-HA as described (Liu et al., 2011). In brief, 2 d.p.t., cells were fixed with 3% paraformaldehyde in PBS for 15 min. and permeabilized with 0.1% Triton-X-100 for 7 min. Cells were incubated with guinea pig anti-CA serum (1:2,000) and mouse anti-HA antibody (Abcam plc, Cambridge, UK, 1:500) diluted in 3% BSA in PBS. As secondary antibodies goat anti-guinea pig Alexa Fluor 568 and goat anti-mouse Alexa Fluor 488 (Invitrogen, Karlsruhe, Germany) were used in dilutions 1:1,600 and 1:2,000 respectively. Nuclei were detected with Hoechst 33342 (1:2000). Images from the center of the cell were taken using a Zeiss LSM700 confocal microscope.

**Protein pull-down assays**

For pull-down-assays of the feA3 variants with FFV-Gag, FFV-Bet, and feA3 proteins, 293 T cells were transfected with 20 μg of pfeA3 expression plasmids encoding the corresponding feA3 forms. 2 d.p.t., cells were lysed in TLE and lysates were cleared by centrifugation. The sample supernatants were then incubated with GST-tagged FFV-Gag, feA322b or -Z3 expression proteins coupled to Glutatione-S-sepharose according to the manufacturers’ instruction (Sigma-Aldrich). The samples were incubated for 60 min. at 4 °C and washed three times with PBS/TX100. For negative control, the samples were incubated with GST alone or GST-coupled BFV Env (Romen et al., 2007). After boiling in electrophoresis sample buffer, samples were subjected to SDS-PAGE and immuno-blotting (Alke et al., 2001).

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**References**

Albin, J.S., Harris, R.S., 2010. Interactions of host APOBEC3 restriction factors with HIV-1 as described (Liu et al., 2011). In brief, 2 d.p.t., cells were fixed with 3% paraformaldehyde in PBS for 15 min. and permeabilized with 0.1% Triton-X-100 for 7 min. Cells were incubated with guinea pig anti-CA serum (1:2,000) and mouse anti-HA antibody (Abcam plc, Cambridge, UK, 1:500) diluted in 3% BSA in PBS. As secondary antibodies goat anti-guinea pig Alexa Fluor 568 and goat anti-mouse Alexa Fluor 488 (Invitrogen, Karlsruhe, Germany) were used in dilutions 1:1,600 and 1:2,000 respectively. Nuclei were detected with Hoechst 33342 (1:2000). Images from the center of the cell were taken using a Zeiss LSM700 confocal microscope.

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