The innate antiviral factor APOBEC3G targets replication of measles, mumps and respiratory syncytial viruses

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The cytidine deaminase APOBEC3G (apolipoprotein B mRNA-editing enzyme-catalytic polypeptide 3G; A3G) exerts antiviral activity against retroviruses, hepatitis B virus, adeno-associated virus and transposable elements. We assessed whether the negative-strand RNA viruses measles, mumps and respiratory syncytial might be affected by A3G, and found that their infectivity was reduced by 1–2 logs (90–99 %) in A3G overexpressing Vero cells, and in T-cell lines expressing A3G at physiological levels. Viral RNA was co-precipitated with HA-tagged A3G and could be amplified by RT-PCR. Interestingly, A3G reduced viral transcription and protein expression in infected cells by 50–70 %, and caused an increased mutation frequency of 0.95 mutations per 1000 nt in comparison to the background level of 0.22/1000. The observed mutations were not specific for A3G [cytidine to uridine (C→U) and guanine to adenine (G→A) hypermutations], nor specific for ADAR (adenosine deaminase acting on RNA, A→G and U→C transitions, with preference for next neighbour-nucleotides U=A>C=G). In addition, A3G mutants with inactivated catalytic deaminase (H257R and E259Q) were inhibitory, indicating that the deaminase activity is not required for the observed antiviral activity. In combination, impaired transcription and increased mutation frequencies are sufficient to cause the observed reduction in viral infectivity and eliminate virus replication within a few passages in A3G-expressing cells.

INTRODUCTION

The cytidine deaminase APOBEC3G (apolipoprotein B mRNA-editing enzyme-catalytic polypeptide 3G; A3G) is a potent inhibitor of retroviruses, hepatitis B virus, adeno-associated virus and transposable elements, and is able to deaminate cytidines in ssDNA replication intermediates leading to cytidine to uridine [C→U(T)] and guanine to adenine (G→A) nucleotide exchanges, a process also referred to as DNA editing or hypermutation (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003; Turelli et al., 2004; Chen et al., 2006; Aguiar & Peterlin, 2008). As an effector molecule of the innate immune response A3G is induced by certain cytokines and detected in human tissues including lung, liver, tonsils, spleen and lymph nodes, where it is expressed predominantly by lymphoid and myeloid cells (Bonvin et al., 2006; Peng et al., 2006; Sarkis et al., 2006; Tanaka et al., 2006; Stopak et al., 2007; Koning et al., 2009; Chen et al., 2010).

The antiviral activity of A3G against human immunodeficiency virus (HIV)-1 was extensively investigated (Sheehy et al., 2002, 2003; Stopak et al., 2003; Yu et al., 2003). In addition to its deaminase activity, A3G exerts antiviral activity in another manner, probably depending on its RNA-binding capacity (Svarovskaia et al., 2004; Zennou et al., 2004; Khan et al., 2005; Newman et al., 2005; Bishop et al., 2006; Wedekind et al., 2006; Burnett & Spearman, 2007; Holmes et al., 2007; Huthoff & Malim, 2007; Iwatani et al., 2007; Bishop et al., 2008; Chelico et al., 2008). It contains two canonical cytidine deaminase domains, of which the C-terminal one is known to actually mediate

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cytidine deamination, whereas the N-terminal domain is involved in RNA-dependent A3G packaging into HIV virions (Huthoff et al., 2009). It is likely that a positively charged pocket in A3G is occupied by RNA, and that this promotes its oligomerization and antiviral activity (Huthoff et al., 2009). Interestingly, A3G can also assemble into large ribonucleoprotein (RNP) complexes present in cytoplasmic microdomains that are associated with RNA regulation, such as mRNA processing bodies (P-bodies) and stress granules (Kozak et al., 2006; Wichroski et al., 2006; Gallois-Montbrun et al., 2007; Parker & Sheth, 2007; Conticello, 2008). These findings suggest that A3G could also potentially affect RNA viruses lacking a DNA replicative intermediate. Interestingly, it has been published that influenza virus, a segmented negative-strand (−) RNA virus, was not affected by A3G (Wang et al., 2008; Pauli et al., 2009).

Here, we investigated whether the replication of three paramyxoviruses may be impaired by A3G by using A3G-expressing Vero cells and T-cell lines for proof of principle. We found that (−) RNA viruses such as measles (MV), mumps (MuV) and human respiratory syncytial virus (RSV) were sensitive to A3G inhibition.

**RESULTS**

**Inhibition of MV, MuV and RSV replication by human A3G**

Constitutively A3G-expressing Vero cells (Fig. 1, + A3G) and control cells (Ctrl) were infected with MV (attenuated and wild-type), MuV and RSV, and the induction of syncytium formation and titres of newly synthesized viruses were analysed. For infection with wild-type MV (tMV<sub>IC<sub>50</sub></sub>-eGFP), target cells were additionally transduced with the appropriate receptor (CD150) expressing retroviral vector 48 h prior to infection. A3G strongly inhibited the syncytium formation induced by the (−) RNA viruses (Fig. 1a–d). Since individual Vero cell lines obtained after transduction with retroviral vectors may eventually produce different results, we used two control cell lines (Ctrl: parental Vero and mock-transduced Vero cells) and two A3G-expressing Vero cell lines (+ A3G: 024-1 and 024-2) for the determination of viral titres (Fig. 1a–d, right panels, lanes 1, 2 and 3, 4, respectively). In both A3G-expressing cell lines, titres of newly synthesized (−) RNA viruses were reduced by 1–2 logs (90–99 %; n = 3, in all cases the reduction was significant with P<0.05). In contrast, replication and infectivity of a DNA virus [herpes simplex virus type 1 (HSV-1); Fig. 1e] was not affected by A3G. The expression of A3G in the Vero cell lines used in this study was controlled by Western blotting (Fig. 1f). A3G was not detected in control cells (Ctrl: mock-transduced Vero cells; Fig. 1f, lane 1), but expressed at high levels in the two A3G-transduced Vero cell lines (024-1 and 024-2; Fig. 1f, lanes 2 and 3). All cell lines proliferated equally (not shown).

In comparison to the expression of A3G in these transduced Vero cells, expression levels in primary human peripheral blood lymphocytes (PBL) were found to be lower (Fig. 1f, lanes 4 and 5). To measure the effect of A3G under more physiological conditions than in A3G overexpressing Vero cells, we used T-cell clones that were modified to constitutively express A3G at levels similar to that found in primary PBL (Fig. 2a). T-cell lines expressing A3G (Fig. 2a, lane 4) and the A3G mutant H257R were used (Fig. 2a, lane 5). The H257R mutation abolishes the catalytic activity of the deaminase (Newman et al., 2005). The production of infectious MV was reduced in both A3G-expressing T-cell lines (Fig. 2b, lanes 2 and 3) in comparison with control cells (Fig. 2b, lane 1) by approximately 1 log. The finding that the H257R mutant inhibited MV replication to a similar extent as wild-type A3G demonstrates that the deaminase activity is not required for antiviral activity of A3G.

In addition, we investigated the inhibition of MV replication in Vero cells freshly transduced with retroviral vectors expressing A3G and the A3G mutants W94L and Y124A [two mutations in the RNA-binding domain, which mediate Alu- and Vif-deficient HIV restriction, and do not alter the expression level of A3G (Bulliard et al., 2009)] and H257R and E259Q [two mutations which abolish the catalytic activity of the deaminase (Newman et al., 2005)]. Transduced cells were infected with MV for 3 days before determining titres of newly synthesized virus (Fig. 3a). A3G and all four mutants reduced virus production by approximately 1 log. The expression of parental A3G and the mutants was controlled by Western blotting (Fig. 3b). Since mutations H257R and E259Q impair the deaminase activity, this again indicates that this activity is not required for the inhibition of MV production.

To demonstrate the specificity of the A3G effect, its expression was inhibited by RNA interference. A3G-expressing Vero cells (024-2) were additionally transduced with retroviral vectors expressing a shRNA against A3G (shRNA-1), a control shRNA with a scrambled sequence (shRNA-sc), and a shRNA against A3G with two mismatched (switched) nucleotides (shRNA-2). In comparison to A3G-expressing control cells (Fig. 3c, lane 2), shRNA-1 efficiently reduced the expression of A3G (Fig. 3c, lane 3), whereas shRNA-sc and shRNA-2 only slightly reduced A3G (Fig. 3c, lanes 4 and 5). The inhibition of infectious MV production in these cells correlated closely with the expression level of A3G (Fig. 3d). Differences between MV titres in A3G-expressing (Fig. 3d, lanes 2, 4 and 5) and shRNA-1-treated cells (Fig. 3d, lane 3) were significant (P<0.04). These data indicate that the inhibition of virus replication is A3G specific.

**Analysis of the A3G effect on viral replication and infectivity**

In order to analyse whether A3G affects viral gene expression and/or manifests in a reduced infectivity of newly synthesized viral particles, we first quantified the effects on viral gene expression. In recombinant (−) RNA viruses, eGFP is expressed from an expression cassette in the viral genome, and therefore reflects the transcriptional
Fig. 1. A3G inhibits the replication of MV, MuV and RSV. Control Vero cells transduced with an empty vector (Ctrl) and Vero cells expressing A3G (+A3G) were infected with MV, MuV, RSV and HSV-1 at an m.o.i. of 0.1. Representative photomicrographs of GFP-positive syncytia were taken after 48 h. Titres (log$_{10}$ p.f.u. ml$^{-1}$) of newly synthesized viruses (right panels) in untreated Vero and mock-treated Vero 023 cells (Ctrl; lanes 1 and 2) and A3G-expressing Vero cells 024-1 and 024-2 (+A3G; lanes 3 and 4) were determined by titration after 3 days. (a) Syncytium formation and titration of attenuated MV (rMV$^{Edt+}_{eGFP}$). Bar, 100 μm. (b) Wild-type MV (rMV$^{IC323}_{eGFP}$) infection of CD150-transduced control and A3G-expressing cells. In this case titres were determined using Vero-hSLAM cells. (c) Syncytium formation by and titration of MuV (rMuVeGFP) and (d) of RSV (rRSV) expressing eGFP. (e) Syncytium formation and titration of recombinant HSV-1-expressing eGFP. All titrations were performed three times ($n=3$). (f) Western blot of cell lysates from mock control Vero cells (Ctrl; lane 1), A3G-expressing cells (024-1, and -2; lanes 2 and 3), and non-stimulated and PHA/IL-2-stimulated primary human PBL (lanes 4 and 5). Lysates were separated by SDS-PAGE, blotted on nitrocellulose, and A3G and GAPDH visualized using rabbit polyclonal antiserum against hA3G and mouse mAb against GAPDH, and peroxidase-conjugated secondary antibodies.
activity of the viral RNA-dependent RNA polymerase (RdRp). Fluorescence signals of both attenuated (not shown) and wild-type MV (Fig. 4a) were reduced in A3G-expressing cells by approximately 50% after 2, 3 and 4 days post-infection. Similar results were obtained for recombinant green RSV (rgRSV; not shown).

In order to measure the inhibitory effect of A3G on the viral RdRp in an additional experimental system, we used an MV mini-genome replication assay as described previously (Rennick et al., 2007). In this assay, the indicator gene expression depends on the activity of the paramyxoviral RdRp (Rennick et al., 2007). A3G inhibited the indicator gene expression by 50–70% (Fig. 4b, lanes 3 and 4; n=3, P<0.01). As control for an inhibitory effect we included the MV-C protein, which has been described as a natural RdRp inhibitor (Bankamp et al., 2005), by co-transfection of an appropriate expression vector. MV-C reduced the indicator gene expression by approximately 85% (Fig. 4b, lane 2).

In contrast to the modest inhibition of the viral transcription by A3G (50–70%), titres of progeny infectious viruses were reduced by 1–2 logs (90–99%; Figs 1 and 2), which implies an additional level of restriction. To investigate this effect more closely, we performed single-step growth experiments, which indicated that the titres of newly produced infectious MV were reduced constantly over time after 2, 3 and 4 days post-infection (Fig. 4c). This inhibitory effect was similar on cell-associated as well as released (supernatant) virus (Fig. 4d).

To estimate the ratio between infectious and defective viral genomes in released particles, we quantified viral transcripts in infected cells and viral genomes in particles released from cells in the absence and presence of A3G by real-time PCR. Differences in copy numbers were calculated according to the ΔΔCt-method. Confirming the results obtained with indicator gene expression, MV-N-specific viral RNAs in infected cells were reduced by A3G by 50–70% (Fig. 5a). Similarly, the copy number of viral genomes in particles released from A3G-expressing cells was reduced by 50–70% (Fig. 5b).

**Viral RNA co-precipitates with A3G**

A3G may exert its antiviral activity against (−) RNA viruses by direct or indirect (via other host factors) interaction with viral RNA. We assessed this possible interaction of A3G with viral RNA using a co-precipitation assay. As a positive control we used cellular hY1 RNA, a family member of L1 (long interspersed nucleotide elements 1)-dependent, non-autonomous hY RNA retroelements (Perreault et al., 2005), which was earlier detected in high molecular mass A3G complexes by RT-PCR (Chiu et al., 2006; Khan et al., 2007). For this experiment, 293T cells were transfected with HA-tagged A3G prior to infection with MV for 3 days, and A3G was immunoprecipitated from cell lysates using HA-specific antibodies. Differences between Ctrl and A3G and H257R cells are significant (P=0.0034 and 0.0032, respectively; n=4).

![Fig. 2. A3G expression in T-cells and inhibition of MV replication.](image-url)
viral RNAs in infected cells. As positive controls RT-PCRs were performed from lysates (Fig. 6a, lane 1), and the specificity of the experiment was confirmed by using cells lacking HA-tagged A3G, from which cDNA-fragments can be amplified from lysates, but not after precipitation with HA-specific antibodies (Fig. 6a, lanes 3 and 4). A Western blot is provided as further control for the specificity of the immunoprecipitation (Fig. 6b).

Enhanced mutation rate, but absence of A3G-specific hypermutations

The results so far do not explain why the infectivity of newly synthesized viral particles from A3G-expressing cells is substantially reduced more than 90%. We therefore investigated if A3G may exert its antiviral activity by causing mutations and/or hypermutations in the viral RNA. First, we assessed whether mutations may accumulate in serial passages of the virus. MV was passaged repeatedly using A3G-expressing (Fig. 7, black and grey bars; 024-1 and 024-2 cells) and control cells (Fig. 7, white bars; Ctrl). For each serial passage newly synthesized virus was titrated, and for the following passage the target cells were infected using the same m.o.i. of 0.1 as initially. In control cells, MV can be passaged indefinitely at titres of approximately $10^{6.5}$ p.f.u. ml$^{-1}$ (Fig. 7a, white bars). In contrast, passaging in A3G-expressing cells could be done for only three subsequent passages until not enough virus could be recovered any longer as the amounts of infectious virus obtained decreased from approximately $10^{4.5}$ in passage one to $10^{3}$ p.f.u. ml$^{-1}$ in passage three (Fig. 7a, black and grey bars).

To determine the mutation frequencies in each passage, RNA was prepared from infected cells, cDNA clones were obtained from RT-PCR amplified portions of MV-N (1063 nt) and MV-M (789 nt) mRNAs [RT primed with oligo-(dT)], and sequenced. For each cell line and passage, and at days 3 and 4 post-infection at least 10 clones were sequenced, altogether 146 clones for MV-N (155198 bp) and 146 clones for MV-M (115194 bp). Already in the first passage (infection with parental virus) the A3G-expressing
cells (Fig. 7b, black and grey bars) gave rise to a larger number of mutations than observed in the absence of A3G (Fig. 7b, white bars), which slightly increased from passage to passage.

Analysis of the overall mutation frequency (Fig. 7c) of all sequenced clones indicated a significant increase in A3G-expressing cells (0.95/1000) in comparison to the mutation frequency in control cells (0.22/1000; n = 98, P < 0.0001). No significant differences were observed between the N and M clones in terms of overall mutation frequency, numbers of transitions (83% of total) versus transversions or synonymous versus non-synonymous mutations (76%). Hypermutations as in the case of Vif-deficient HIV-1 (Lecossier et al., 2003) were not observed. Most frequently we found A → G and U → C mutations, and less frequently A3G deaminase-specific C → U(T), or G → A mutations (Fig. 7c, arrows). Thus, our data indicate that the frequency of mutations in the presence of A3G is increased significantly, while the mutations are not specifically

![Fig. 4](image_url)

**Fig. 4.** Impact of A3G on viral gene expression and infectivity of progeny viruses. Viral protein expression (a) was quantified using the fluorescence reader detecting eGFP autofluorescence of wild-type MV (rMVIC323eGFP) infected cells (n = 3). The fluorescence reader measures a standardized area of the culture dish covered by adherent living cells. An MV mini-genome replication assay (see Methods) was used to quantify the RdRp activity (b) in control (Ctrl) cells without and with the MV-C protein (+MVC) as polymerase inhibitor (lanes 1 and 2), and in A3G-expressing cells (024-1 and 024-2; lanes 3 and 4; n = 3). Virus growth (c) was analysed in single-step growth experiments using wild-type MV (rMVIC323eGFP) at an m.o.i. of 0.1 to infect control and A3G-expressing cells as indicated. Cell associated and supernatant (d) MV (rMVEdtageGFP) was analysed separately after 3 and 4 days post-infection in control and A3G-expressing cells as indicated.
caused by its cytidine deaminase activity. These results were confirmed by sequencing amplified and cloned cDNAs from purified viral particles (Fig. 7d; N and M fragments as described; RT primed with random hexamers). In viral particles produced by A3G-expressing cells we found a frequency of 0.63/1000, and a similar pattern of transition (70%) versus transversions; synonymous and non-synonymous (86%) mutations as in transcripts from the cytoplasm of infected cells (Fig. 7d, black and grey bars). The background mutation frequency in control virus preparations propagated in the absence of A3G was significantly lower with 0.11/1000 (Fig. 7d, white bars; n=40, P<0.001).

DISCUSSION

Using A3G overexpressing interferon-deficient Vero cells and T-cells expressing A3G at physiological levels we demonstrated that A3G reduces infectious virus production of the three tested (−) RNA viruses MV, MuV and RSV by more than 90%. Furthermore, using MV as a model virus, we found that (i) viral transcripts are reduced by A3G by about 50–70%, (ii) that A3G interacts directly or indirectly with viral mRNAs, and (iii) that the frequency of mutations in viral transcripts is enhanced independent of the deaminase activity of A3G.

The frequency of nucleotide replacements increased in the presence of A3G from 0.2 to 0.95/1000, while qualitatively the pattern of mutated nucleotides in control cells and A3G-expressing cells appeared similar. Transitions of A→G were the most frequent, followed by T→C, G→A and C→T or C→A, confirming that this is not a consequence of specific A3G deaminase activity. Other deaminases, adenosine deaminases acting on RNA (ADARs), target double-stranded regions in RNA and produce clusters of U→C or A→G transitions. Such clustered hypermutations have been observed predominantly in matrix genes of MV sequences from brains of patients suffering from subacute sclerosing panencephalitis (SSPE) (Cattaneo et al., 1988; Baczko et al., 1993; Hotta et al., 2006). However, since we did not detect hypermutations or clustered U→C or A→G mutations, while other mutations were almost as frequent, and since 5′ nearest neighbour-nucleotides (not shown) were not specific for ADARs (Suspenne et al., 2011), our findings do not support the view that these mutations are caused by ADARs. Since the frequency distribution of mutated nucleotides was proportional to the mutational pattern detected in the absence of A3G, our findings suggest that in the presence of A3G the fidelity of the viral polymerase is impaired and its intrinsic error rate is enhanced.

In comparison to G→A hypermutations as observed in HIV patients (Janini et al., 2001), and in tissue culture using Vif-deficient HIV-1 [approx. 4 or 10–20 mutations/1000 bp, respectively; (Bishop et al., 2004)], or U→C hypermutations found in MV sequences from SSPE patients [up to 50% of U in the matrix gene (Bass, 2002)], the mutation frequency we found (0.95/1000) is low. The pattern of mutations indicates a normal distribution of transitions versus transversions. The type of mutations appears to have a random distribution resulting in approximately 80% non-synonymous mutations. If we extrapolate a mutation frequency of 0.63–0.95/1000 to the
genome size of approximately 16000 nt of paramyxoviruses this leads to approximately 10–15 randomly distributed mutations per viral genome (of which approximately five are situated within the ORF of the polymerase), resulting in approximately 7–12 aa exchanges or introductions of stop codons, a fraction of which will render viral proteins non-functional. This is probably more than the paramyxoviral genome can tolerate for further successful replication and explains the lack of fixation of these mutations in virus stocks passed for a number of times in the A3G-expressing cells. The observed mutation rate may already be sufficient to explain the moderate decrease of viral gene expression in the first round of infection as demonstrated. Slightly reduced but nevertheless significantly enhanced mutation frequencies in the purified particles potentially explain that only a small percentage of the viral particles is functional and infectious. Taken together, the observed effects are sufficient to explain the antiviral activity of A3G against (-) RNA viruses leading to a 90–99 % reduction of viral titres per passage.

A3G is distributed over the cytoplasm and localizes to P-bodies, stress granules, Staufen-containing RNA granules, or Ro-RNPs, large RNP complexes present in cytoplasmic microdomains that are associated with RNA regulation (Kozak et al., 2006; Wichroski et al., 2006; Gallois-Montbrun et al., 2007; Parker & Sheth, 2007). P-bodies constitute specialized compartments where non-translating mRNAs accumulate and are subject to storage and degradation. Biochemical analyses demonstrated that A3G interacts with other P-body proteins that function in cap-dependent translation, translation suppression, RNA interference-mediated gene silencing and decapping of mRNA (Wichroski et al., 2006). Investigating possible interactions of A3G with viral components, we detected co-precipitated viral RNA. The finding that only some primers were able to amplify viral RNA bound to immunoprecipitated A3G may be due to the detection limit of the assay, although we cannot also exclude that a sequence-specific binding may occur as observed with other APOBECs (Navaratnam et al., 1995; Gallois-Montbrun et al., 2008).

Interestingly, viral mRNAs found in viral factories (N-, P- and L-mRNAs) were not found to interact, whereas those mRNAs being translated elsewhere (M-, H- and F-mRNAs) did interact with A3G. In addition, this interaction may possibly be exerted by other host factors involved in RNA processing. The interaction of A3G with host proteins (Gallois-Montbrun et al., 2007) and a cell type-specific expression of factors required for the antiviral activity of A3G has already been observed (Han et al., 2008) and requires further investigations.

**METHODS**

**Cells and viruses.** Human embryonal kidney 293T cells, African green monkey Vero cells, mock control Vero (Vero-118) cells transduced with the vector pcMS28 lacking A3G cDNA (023), Vero cells transduced with pcMS28 expressing human A3G (024-1 and...
024-2), and Vero-hSLAM cells were cultivated in standard Dulbecco's modified Eagle's medium (DMEM) medium. Transduced Vero cells were grown under selection pressure with 5 μg puromycin ml⁻¹ as bulk culture (024-2), or as clone achieved with limited dilution cloning (024-1) as described previously (Gallois-Montbrun et al., 2007). CEM-SS-derived T-cell lines were maintained in RPMI medium containing 10% FCS.

Recombinant wild-type MV rMV1312eGFP (Hashimoto et al., 2002) and the attenuated MV rMVEdtageGFP (Duprex et al., 1999) were propagated using Vero-hSLAM cells. rgRSV [a kind gift from P. Collins and M. Peeples, Bethesda (Hallak et al., 2000) and C. Krempel, Würzburg], and the gEFP-expressing recombinant MuV rMuVeGFP [derived from the strain Jeryl Lynn 2 (Chambers et al., 2009)] were propagated using Vero cells. Recombinant HSV-1-expressing GFP [HSV1(17+)-Lox-PMCMVGFP], a kind gift from B. Sodeik, Hannover, was propagated using BHK cells. Vaccinia virus MVA-T7, a gift from G. Sutter, Langen, was propagated using BHK cells. For assessing the effect of A3G on viral replication, A3G-expressing cells in six-well plates were infected at an m.o.i. of 0.1, and virus was harvested after indicated times by freezing and thawing the complete culture, if not indicated then supernatant and cell-associated virus was harvested separately.

**Fig. 7.** A3G effect on viral titres in serial passages and analyses of mutations. (a) Control cells (Ctrl; open bars) and Vero cells expressing A3G (024-1, black bars; 024-2, grey bars) were infected with MV (rMVEdtageGFP) at an m.o.i. of 0.1 and virus harvested after 3 and 4 days. Virus preparations were titrated using Vero cells. Control and A3G-expressing cells were infected again (m.o.i. of 0.1) for a second passage. The same procedure was repeated for a third passage. Viral titres of passages 1–3 are presented. (b) At least 10 clones of MV-N (1063 nt) and MV-M (789 nt) fragments of each viral passage were ampliﬁed by RT-PCR, subcloned by TOPO-TA cloning and sequenced. The number of mutations per 1000 bp in each passage of virus is presented. (c) Derived from intracellular transcripts, 146 N- and 146 M-clones representing 270 392 sequenced nucleotides were analysed in total. The cumulative specific mutations are presented. Black and grey bars: specific mutations in A3G-expressing 024-1 and 024-2 cells; white bars: specific mutations in control cells. Arrows are explained in the text. (d) Cumulative mutations as sequenced from 20 N- and 20 M-clones derived from RNA of puriﬁed viral particles from the supernatant of infected A3G-expressing (black and grey bars; 74 080 nt sequenced) and control cells (white bars; 37 040 nt sequenced).
Cloning and sequencing. Parts of MV-N and MV-M cDNA from infected cells were cloned into pGEM-T-Easy (Promega) using primers MV-N-fw (5’-AAGGCTTACGAGGTGTTTCC-3’) and MV-N-rv (5’-AACGATGTGCTTGATCCGGAGTGGG-3’), and MV-M-fw (5’-ATACACACGCTGCTGTGTC-3’) and MV-M-rv (5’-GGGTGGTTTGGATCGG-3’). A 1063 bp fragment of MV-N was sequenced using the primers MV-N-seq1 and -2 (5’-TTCGCGCCATTTGCATTTATTAGGACCTGACTG-3’) and MV-M-seq1 and -2 (5’-TTCCGTGGTGTATATTGCAGCCACAGATTACCC-3’) and MV-M-rv (5’-AGAGAACCTCTGGTACCACCG-3’).

The murine retroviral vector pMS28 (P. Gallois-Montbrun et al., 2005) was used as a backbone for cloning and expression of hA3G (pRH024), A3G mutants and hSLAM (pcMSSLAM) ORFs as described previously (Reuter et al., 2006), and genomic primers were used as described previously (Plumet & Gerlier, 2005). Real-time PCR was performed using a LightCycler 2.0 real-time PCR System (Roche) in 20 μl consisting of 10 μl QuantiTect SYBR Green (Qiagen), 5 μl cDNA and 20 μl of each primer. The cycling conditions were 50 cycles with 15 s at 94 °C, 30 s at 56 °C and 20 s at 72 °C after a preheating step of 15 min at 95 °C. A plasmid containing the complete MV Genome [p(+)MVNS; (Radecke et al., 1995)] was used for a standard curve. Analysis of the melting curve indicated the presence of only a single product in each reaction. The efficiency (E) of each PCR primer pair was determined from the slope of the standard, according to the equation E = 10(–1/slope). Efficiencies were 90–98 %. Relative quantification of the transcripts was carried out according to the ΔΔCt method described previously (Livak & Schmittgen, 2001).

MV mini-genome replication assay. Plasmids for the MV mini-replicon assay were used as described previously (Renneck et al., 2007) or newly cloned [p(–)MV-DI-eGFP]. Briefly, Vero cells were grown to 80 % confluency in six-well trays, rinsed with DMEM without FCS and infected with MVA-T7 at an m.o.i. of 2 for 45 min. After removal of the inoculum, a mixture containing plasmids encoding MV N, P and L (pEMC-Na (1.2 μg), pEMC-Fa (1.2 μg), pEMC-La (0.4 μg) and the mini-genome p(–)MV-DI-eGFP (4 μg) was transfected using PEI (25 K; Polysciences) and 1 ml DMEM was added to each well. After 3 h incubation at 37 °C, the supernatant was replaced with DMEM containing 5 % FCS. The eGFP expression was quantified after 30 h using flow cytometry. The MV-C expressing plasmid pCG-DUBFLAG (a kind gift from E. Millar, Belfast) was co-transfected as control.

A3G-RNA-binding assay. 293T cells (1.5 × 105) were transfected with 3 μg pA3G-HA (Huthoff et al., 2009) using PEI and infected with rMV at an m.o.i. of 0.1. After 16 h medium was replaced, and after two additional days cells were lysed in 500 μl M-PER reagent (Pierce) containing 0.1 % RNasin RNase inhibitor (Promega) μl−1. Lysates were cleared by centrifugation and split into two aliquots of 250 μl. With one aliquot, immunoprecipitation was performed using ProFound mammalian HA tag IP/Co-IP kit (Pierce) following the manufacturer’s instructions, except for washing the beads three times with RNA–protein-binding buffer (Khan et al., 2007). Following immunoprecipitation or protein lysis, RNA was isolated using the GenElute mammalian total RNA miniprep kit (Sigma), and processed for RT-PCR using RevertAid M-MuLV reverse transcriptase and a mix of oligo(dT)12/18 random hexamer primers. PCR was performed using Taq polymerase (Fermentas) and specific primers for hY1 RNA (Chiu et al., 2006), MV-encoding gfp (GF-P-fw 5’-GCGAGGCTGACCTACAGGAC-3’ and GF-P-rv 5’-GGGTTTCTGCTGTTGATGCAG-3’), MV-N (5’-CAACACTTGTGCTTGATCA-3’), MV-P (5’-GGGTTTCTGCTGTTGATCA-3’) and MV-L (5’-GGGTTTCTGCTGTTGATCA-3’) or anti-GAPDH mAb (clone 1D4; Acros), and HRP-conjugated secondary antibodies (1:2000; Immunootech). Signals were visualized using the enhanced chemiluminescence system (Amersham).
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