The innate antiretroviral factor APOBEC3G does not affect human LINE-1 retrotransposition in a cell culture assay

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Running title: APOBEC3G and LINE-1 retrotransposition
SUMMARY

APOBEC3G is an innate intracellular antiretroviral factor that can inhibit viral retroelements such as retroviruses and hepadnaviruses. However it is unknown whether it can act on non-viral substrates. Retrotransposons are transposable elements that cumulatively account for about one third of the human genome. They are commonly classified in long terminal repeat (LTR) retrotransposons, which are strongly homologous to retroviruses, and non-LTR retrotransposons also known as L1 elements or LINE-1 (long interspersed nucleotide element-1) elements. Most of the L1 elements are defective and only a small number are very active in vivo but they are responsible for nearby all of the retrotransposition in the human population. The cloning of active human L1 elements has allowed the development of tissue culture-based assays for measuring their retrotransposition potential. We used such an assay to demonstrate that APOBEC3G, which impairs the replication of exogenous retroelements, does not affect the replication of endogenous L1 retrotransposons.

INTRODUCTION

Human APOBEC3G (Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G), also known as CEM15, is a cytidine deaminase that can block the replication of a wide array of retroelements (1-3). Packaged in retroviral particles, APOBEC3G (APO3G) associates with the reverse transcription complex where it deaminates cytosine residues to uracil in the growing minus-strand viral DNA. These dU-rich transcripts are either degraded or yield G-to-A hypermutated hence largely non-functional proviruses (1,2,4). APOBEC3G is countered by the Vif (virion infectivity factor) protein of lentiviruses, which associates with the cellular enzyme to prevent its virion incorporation and trigger its proteasomal degradation (1,2,5-8). In the absence of Vif, the innate
antiviral protein can inhibit human and simian immunodeficiency viruses (HIV and SIV, respectively), equine infectious anemia virus (EIAV), and the gammaretrovirus murine leukemia virus (MLV). In addition, APOBEC3G can block the replication of hepatitis B virus (HBV), a hepadnavirus whose life cycle also includes reverse transcription (3). In this case, however, one observes an inhibition of viral pregenomic RNA packaging, and thus of viral DNA synthesis, rather than the lethal editing of viral reverse transcripts.

Retrotransposons are commonly classified as long terminal repeat (LTR) and non-LTR retrotransposons (9). The Ty1/3 elements and copia elements are LTR retrotransposons of yeast and Drosophila melanogaster, respectively, and actively participate in the genome remodeling of these organisms. In higher species, LTR retrotransposons, also known as endogenous retroviruses, are most commonly defective and usually cannot spread from cell to cell. Evidence exists for ongoing LTR retrotransposition in the mouse, but no active LTR retrotransposon has so far been isolated in humans, even though human endogenous retroviruses (HERVs) make up roughly 7% of the genome. Yet the presence of human-specific HERV proviruses and their absence in corresponding loci of other primates indicate that a subset of these LTR retrotransposons has been active during the recent evolution of hominoids.

L1 elements occupy close to 20% of the human genome (10). Their vast majority is defective due to truncations or rearrangements, but three to four thousand human L1s are full-length, amongst which an estimated 80-100 in the average human are active (11). By comparison, the mouse genome contains up to three thousand active L1 elements. L1 retrotransposition accounts for several genetic disorders in humans, including cases of haemophilia A, Duchene muscular dystrophy, type-2 retinitis pigmentosa, ß-thalassaemia and chronic granulomatous disease (12-16). Active human L1 retrotransposons have been cloned and tissue-culture-based assay has been developed to compare their
retrotransposition ability (11,17,18). Because L1 elements replication is dependent on reverse transcription and because, in the case of retroviruses, APOBEC3G target is the growing minus-strand DNA which is formed during reverse transcription, we used this culture-based assay to determine if APOBEC3G can interfere with L1 retrotransposition.
EXPERIMENTAL PROCEDURES

Plasmids and viral stocks. Wild-type and vif-defective HIV-1 proviral clones were previously described (19,20). The MLV-based pNG-empty and pNG-APOBEC3G-HA vector system, and the plasmid expressing a HA-tagged form of APOBEC3G were a kind gift from M. Malim (University College, London, UK). The constructs pL1RP-EGFP, pL1-ac002980-EGFP and pL1RP-(JM111)-EGFP were previously described (11,17). The MLV vector stocks and HIV viral stocks were produced by transient transfection of 293T cells or 143BTK cells respectively, with Fugene (Roche) (see www.tronolab.unige.ch for details). Infections and titrations were performed as previously described (21). Virion infectivity was derived by dividing the infectious titer by the amount of physical particles (as measured with reverse transcriptase activity).

Cells and assay for retrotransposition activity. The human 143BTK- osteocarcinoma cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. To obtain stably APOBEC3G-HA- or control-expressing 143BTK-, cells transduced with the MLV- based vectors were selected with neomycin (400µg/ml). These neomycin-selected cells were transfected independently in triplicates with the retrotransposons expressing vectors, grown overnight and selected with puromycin as previously described (11). EGFP expression was quantified regularly by FACS analysis for 21 days. A gated sample of 10,000 live cells was analyzed at each time point.

Protein analyses. Indirect immunofluorescence was performed on 143BTK- neomycin and -puromycin selected cells as previously described (22), using the monoclonal HA.11 antibody (BAbCO) to detect APOBEC3G-HA and TOTO (Molecular probe) to detect the
nuclei, as recommended by the manufacturers. For western blot analyses, cells were lysed with RIPA buffer and subjected to standard SDS-PAGE. For HA- and PCNA- specific detection, the mouse monoclonal 3F10-peroxydase-conjugated antibody (Roche) and the mouse monoclonal anti-PCNA (Oncogene Research Products, Boston, MA) were used respectively.

**Sequencing.** DNA was extracted with the DNeasy kit (Qiagen) and submitted to amplification (using high fidelity Pfu Turbo polymerase, Stratagene) with primers specific for EGFP and encompassing the intron (PB.retro1 sense, BamHI-ATGATGGATCCAGCTGGACGGCGACGTAAAC; PB.retro2 antisense, TATAGAATTCTCCATGCGAGGTGTGATCCC-EcoRI). The PCR products were cloned and independent clones were sequenced.
RESULTS AND DISCUSSION

L1 retrotransposons have been cloned and in vitro assays have been developed to measure their capacity to retrotranspose. Here we used the pL1RP-EGFP plasmid which contains a full-length active human L1 element with a retrotransposition detector cassette inserted in its 3’ untranslated region (Fig. 1). This cassette comprises a CMV promoter driving expression of an EGFP reporter separated by a γ-globin intron in the antisense orientation. EGFP expression can only occur when the intron is removed by splicing during a retrotransposition event. A puromycin resistance gene further allows the selection of cells transfected with this plasmid. Construct pL1-ac002980-EGFP has a similar organization, being derived from a second human L1 element previously found to have higher levels of retrotransposition activity than pL1RP-EGFP (11). The pL1RP-(JM111)-EGFP encodes an L1 element containing missense mutations that were previously shown to abolish retrotransposition (18).

We took advantage of this system to ask whether APOBEC3G could interfere with human L1 retrotransposition. The 143BTK- cell line is a substrate very conducive to L1 retrotransposition, but we first needed to ascertain that it could reveal APOBEC3G action. For this, we established 143BTK- derivatives that stably express the cytidine deaminase owing to transduction by an APOBEC3G-containing retroviral vector (Fig. 2A). Vif-defective HIV-1 produced by transfection from these but not control cells exhibited levels of infectivity that were about fifty-fold lower than wild-type, very similar to those measured for ΔVif virions produced in these cells when an APOBEC3G-expressing plasmid is co-transfected together with the viral DNA (Fig. 2B). We thus transfected the APOBEC3G-expressing and control 143BTK- cells with pL1RP-EGFP, pL1-ac002980-EGFP and pL1RP-(JM111)-EGFP, the latter serving as a negative control. Puromycin selection was then applied. Expression of APOBEC3G-HA was verified in
these doubly selected cells by western blotting (Fig. 3A) and the percentage of GFP-positive cells was monitored over time by FACS analysis (Fig. 3B). Cells transfected with the retrotransposition-incompetent pL1_{RP}-(JM111)-EGFP plasmid remained negative throughout the study. With pL1_{RP}-EGFP, 6% of the cells were GFP-positive at day 7, and this fraction increased to 9% at day 21, reflecting ongoing retrotransposition. As previously described (11), pL1-aco02980-EGFP was significantly more active, with 8 and 14% GFP-positive cells at days 7 and 21, respectively. However, in no case were these results affected by the presence of APOBEC3G. Accordingly, L1 elements which have lost the intron during retrotransposition have been specifically cloned and sequenced but no G-to-A mutations were found (data not shown). Because expression of the GFP marker from the pL1_{RP}-EGFP and pL1-aco02980-EGFP constructs requires transcription, splicing, reverse transcription and integration of the GFP cassette together with the full preservation of its coding sequence, we conclude that APOBEC3G does not interfere with any of these steps. Accordingly, mechanisms put into play by the cellular enzyme in the control of exogenous retroelements such as lentiviruses, gammaretroviruses and hepatitis B virus do not affect human L1 retrotransposition. Specific features of L1 elements may explain their resistance to APOBEC3G action. Indeed, L1 retrotransposition is based on target-primed reverse transcription, a process in which the endonuclease-cleaved target DNA serves as a primer from which complementary DNA is made using the polyadenylated L1 RNA as a template (23,24). Synthesis of the L1 DNA thus takes place in the nucleus, whereas APOBEC3G is a predominantly cytoplasmic protein. Nevertheless, the L1 ribonucleoprotein complex thought to act as an intermediate in retrotransposition is assembled in the cytoplasm, therefore one can assume that it does not carry the determinants that recruit the cytidine deaminase in its retroviral and perhaps hepadnaviral counterparts.
These results strongly argue against a role for APOBEC3G in the control of L1 retrotransposition. However, they do not allow any conclusion regarding a possible effect of the cellular enzyme on endogenous retroviruses. Many HERVs carry strong homology with gammaretroviruses such as MLV, a virus sensitive to the inhibitory effect of APOBEC3G. Short of the identification of active HERVs and of a tissue-culture based assay for measuring their retrotransposition ability, a careful comparison of the A/G content of HERVs with that of related exogenous retroviruses might give hints regarding a possible role for APOBEC3G-mediated editing in HERV control. As well, parallel experiments in other species may be helpful, since active endogenous retroviruses may exist in other mammals, for instance the mouse.

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**FOOTNOTES**

The abbreviations used are: APOBEC3G, apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G; APO3G, APOBEC3G; LINE-1, long interspersed nucleotide element-1; L1, LINE-1; Vif, virion infectivity factor; HIV-1, human immunodeficiency virus 1; SIV, simian immunodeficiency virus; MLV, murine leukemia virus; HBV, hepatitis B virus.
FIGURE LEGENDS

Fig. 1. The retrotransposition system.
The plasmids used contain a puromycin resistance gene allowing selection of the transfected cells and a retrotransposition competent, full-length human L1 element with an EGFP cassette inserted in its 3’ untranslated region. This cassette comprises a CMV promoter driving an EGFP reporter interrupted by a γ-globin intron in the antisense orientation. EGFP can be expressed only after transcription, splicing of the intron, reverse transcription and integration of the cassette.

Fig. 2. Functional analysis of the 143BTK- cells expressing APOBEC3G.
(A) The 143BTK- cells were transduced with the MLV-based pNG-empty or pNG-APOBEC3G-HA vectors and selected with neomycin. Expression of APOBEC3G-HA in the selected cells was verified by immunofluorescence with anti-HA antibody (green). Nuclei were stained with TOTO (blue) (B) The neomycin-selected 143BTK-cells were transfected with wild-type HIV, ΔVif HIV or ΔVif HIV plus APOBEC3G-HA expressing plasmids. The viral supernatants produced were collected and infectivity was measured in a single round assay as described in materials and methods (and expressed as transducing unit per amount of reverse transcriptase activity).

Fig. 3. Retrotransposition rate in the presence of APOBEC3G.
(A) Western blot analysis of extracts from the neomycin selected APOBEC3G-expressing and control 143BTK- cells, which have been transfected with pL1RP-EGFP, pL1-ac002980-EGFP or pL1RP-(JM111)-EGFP and selected with puromycin. Antibodies against HA and PCNA were used (B) The retrotransposition rate was
monitored with time by FACS analysis, in the above described neomycin and puromycin selected cells.
Fig. 1

Transfection and puromycin selection

Transcription

Removal of intron by splicing

Reverse transcription and integration

EGFP expression

FACS analysis
Fig. 2
Fig. 3

A

| PNG empty | PNG APO3G-HA |
|-----------|--------------|
| 1         | 2            |
| 3         | 1            |
| 2         | 3            |

APO3G-HA
PCNA

B

- JM111
- RP
- ac002980
- JM111 + APO3G
- RP + APO3G
- ac002980 + APO3G

GFP+ cells (%)
days post-transfection
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