MLV glycosylated-Gag is an infectivity factor that rescues Nef-deficient HIV-1

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Optimal infectivity of HIV-1 virions requires synthesis of the HIV-1 regulatory protein Nef in some producer cells but not others. A survey of 18 lymphoid cell lines found that Nef was dispensable in three, each of which harbored gammaretroviruses. Nef-dependent cell lines were rendered Nef-independent by a cell-free supernatant from the independent lines or by transfection of cloned murine leukemia virus (MLV). Analysis of MLV deletion mutations identified glycosylated gag (glycogag) as the factor that rescues Nef-defective HIV-1 virions. Glycogag was also demonstrated to be required for the infectivity of MLV virions produced in lymphoid cells. Direct comparison of Nef and glycogag revealed identical dependence for activity on Env-pseudotype and producer cell type. The two proteins colocalize within cells, and both increase the yield of viral cDNA in target cells. The functional similarity of Nef and glycogag is a compelling example of convergent evolution in which two structurally unrelated proteins provide a function necessary for virion infectivity in lymphoid cells.

Nef is a myristoylated protein encoded by HIV-1, HIV-2, and SIV, crucial for virus replication in vivo and rapid AIDS progression (1–3). It performs a remarkable array of activities by exploiting many of its surfaces to interact with several cellular molecules. By interacting with proteins implicated in intracellular trafficking, it modulates cell surface expression of numerous molecules, including the receptor CD4 (4, 5) and MHC-I (6). Alleles derived from most SIV isolates also down-regulate the TCR/CD3 complex (7–9). In addition, Nef alters the activation threshold of lymphocytes (10–12) by interacting with protein kinases (13–16) and modulates apoptotic signals (10, 17).

Nef also has a positive effect on the infectivity of virions (18, 19), a function which remains mechanistically unexplained. Although the ability to down-regulate CD4 can contribute to the effect of Nef on infectivity (20), this activity is visible by using CD4-negative producer cells and was shown to be independent from other Nef effects (18, 21–23), it requires its expression in virus-producing cells and is manifested at an early step of the infection process of target cells (18, 22, 24–26). Nef might play a crucial role during penetration of retroviral cores into the cytoplasm (27). Accordingly, HIV-1 pseudotyped by vesicular stomatitis virus G (VSV-G), which relies on endosomal uptake and, therefore, might enhance cytoplasmic delivery, does not require Nef (28, 29). Although found in virus particles, recent data indicate that Nef itself might not function as a virion protein (30, 31), suggesting that it could induce a yet unknown modification of the particle. The effect of Nef on infectivity was shown to depend on dynamin 2 and clathrin activities in producer cells (32) and on a di-leucine motif critical for the interaction with the clathrin adaptor complexes AP2 (33, 34). The biogenesis and/or trafficking of intracellular vesicles in virus producing cells might therefore play a crucial role in modulating virion infectivity.

Most gammaretroviruses encode an accessory protein (gPr80 or glycogag) from unspliced RNA via an alternative CUG initiation codon upstream and in-frame with the standard Gag polyproteins (35–39). As a result, a leader sequence (88 amino acids in MLV provirus genome (MoMLV)) is added to the N terminus of conventional Gag. The protein encoded has a type II transmembrane topology, with the conventional Gag residues being extracellular or located in the lumen of the ER, and glycosylated. Mature gPr80 is proteolytically cleaved, and only half of the conventional Gag sequence remains attached to the integral transmembrane protein (40). Although not strictly required for virus replication in vitro, gPr80 is crucial in vivo for sustained virus replication and disease progression (41–47). The mechanisms engaged by gPr80 to promote virus replication remain largely unknown. However, a recent report has revealed that gPr80 affects release of both MLV and HIV-1 by facilitating budding from lipid rafts (48).

In this study, a screen of 18 producer cell lines identified three lymphoid cell lines capable of generating HIV-1 that does not require Nef for maximal infectivity. Glycosylated gag expressed from gammaretrovirus genomes harbored by these cell lines was found to functionally replace the infectivity function of Nef, unveiling a role for glycogag as an infectivity factor.

Results

Nef Is Not Required for Optimal Infectivity of HIV-1 Derived from Three Human Lymphoid Cell Lines. The ability of Nef to enhance HIV-1 infectivity was studied by using a panel of 18 human cell lines of hematopoietic origin, including sublines derived from common progenitors (Table S1). Nef-positive (HIV-1WT) and Nef-defective (HIV-1Nef-) virions limited to a single cycle of replication were produced by transient transfection and inoculated onto TZM-bl reporter cells (Fig. L4). Nef did not enhance viruses derived from Jurkat J6 (JJ6), CEM/A3.01-F7, and DG75-UV cells (here defined as “Nef-independent”), but increased (6- to 40-fold) the infectivity of HIV-1 derived from the remaining 15 cell lines (defined as “Nef-dependent”), which include other Jurkat, CEM, and DG75 sublines. The absence of Nef activity on virus harbored by JJ6 is in striking contrast with the 25- to 40-fold enhancement observed on HIV-1 derived from the parental cell line (JM) or from other Jurkat sublines (JE6.1, JD1.1, JTA) with common genomic background between JTA and JJ6 was confirmed by sequencing the region spanning the V-D-J junction of the T cell receptor gene (SI Text), revealing that the Nef phenotype can be profoundly different, despite identical genetic background of the producer cells.

Measurement of the absolute infectivity of viruses released by sister sublines showed that HIV-1 generated by Nef-independent cell lines is equal to, or more infectious than, HIV-1WT released by Nef-dependent cell lines (Fig. 1B). This evidence indicates that JJ6, CEM/A3.01-F7, and DG75-UV cells have either

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gain Nef-like infectivity function or lost an inhibiting factor that can be counteracted by Nef.

**A Gammaretrovirus in Producer Cells Substitutes for Nef.** JJ6 and CEM/A3.01-F7 cells were recently found to release xenotropic MLV and gibbon ape leukemia virus (49). A xenotropic MLV had also been detected in DG75-UW cells (50). Using a PCR-based reverse transcriptase assay (51), no RT activity was detected in culture supernatant of other cells used in this study, indicating the presence of gammaretroviruses as a distinctive property of Nef-independent producer cell lines. If the absence of the Nef effect is caused by another virus in the producer cells, the Nef-independent phenotype should be transferable to Nef-dependent cell lines. JTAg cells were therefore exposed to cell-free JJ6 growth medium and cultured until levels of RT activity released indicated that the JJ6 MLV-X had propagated. The newly infected cell line (JTAg/JJ6) produced HIV-1 no longer released indicated that the JJ6 MLV-X had propagated. The absence of the Nef phenotype can therefore be transferred from one cell line to another.

To demonstrate that the presence of a gammaretrovirus genome in producer cells abrogates the Nef requirement, HIV-1 was produced from JTAg cells cotransfected with a plasmid containing the MoMLV (41). The MLV provirus in producer cells enhanced the infectivity of the HIV-1 wt 60-fold and that of HIV-1 nef only 2.5-fold (Fig. 2B). Infectivity of virus particles pseudotyped with VSV-G, which abrogates the Nef dependence, were only minimally responsive to the effect of MLV (Fig. 2C), indicating that the gammaretrovirus genome is active on HIV-1 particles that require Nef.

**5′ Region of MLV Genome Is Essential for the Effect on HIV-1 Infectivity.** To map the MLV determinant that has Nef-like activity, frameshifts and deletions were introduced by using restriction sites in the MLV provirus to disrupt the coding capacity of MLV ORFs. The mutated genomes were tested for their ability to rescue the defective infectivity of HIV-1 nef (Fig. 3A). The activity was not affected by mutations disrupting env or 92% of gag. In contrast, activity was abolished by deletions close to the 5′-end of the genome, indicating that the region crucial for the effect on HIV-1 infectivity requires no more than 100 codons of the ordinary Gag protein and might include sequences upstream of gag.

Most gammaretroviruses encode a glycosylated transmembrane Gag molecule (glycogag or gPr80) translated from a CUG initiation codon upstream and in-frame with gag (35–39). To test the possibility that glycogag is capable of rescuing the infectivity defect of HIV-1 nef, a mutant genome unable to encode gPr80 (MLV53) was obtained by changing the “CUG” start codon to “CA”; this mutation introduces both a nucleotide change and a frameshift. MLV53 in producer cells was unable to rescue the infectivity of HIV-1 nef (Fig. 3B), proving that gPr80 is required for the effect on HIV-1.

A vector encoding a minimal active glycogag molecule (based on Fig. 3A) truncated at residue 189 and fused to the HA peptide at its N terminus (HA-gg199, Fig. 3C) had indistinguishable activity from full-length MLV (Fig. 3C), demonstrating that other MLV gene products are dispensable for this function. A further C-terminally truncated molecule (HA-gg139), retaining only 10 aa of the extracellular tail, maintained significant ability to rescue HIV-1 nef (Fig. 3C), indicating that the extracellular domain is not strictly required for the activity. Of note, although glycogag
expression had a robust effect on HIV-1 infectivity, it did not affect the efficiency of virus particle release.

**Glycogag Is an Infectivity Factor for MLV.** To investigate the function of gPr80 in the context of MLV infectivity, MLV^WT^ and MLV^gg^-restricted to a single cycle of replication were produced by using JTAg cells. To allow efficient infection of human cells, virions were initially pseudotyped with either xenotropic or amphotropic Env and inoculated onto NIH3T3 cells followed by immunofluorescence staining of CA (Fig. 4A and Fig. S1). Loss of glycogag expression did not adversely affect particle production but caused a 15-fold decrease of infectivity, indicating a crucial role of gPr80 for amphotropic and xenotropic infection pathways.

The activity of gPr80 was then tested on MLV pseudotyped with the ecotropic Env (MLV-E). Glycogag was not required for infectivity of MLV-E inoculated on NIH 3T3, HT1080, or HeLa cells expressing the receptor for ecotropic MLV (mCAT1), but a 3-fold and 8-fold reduction was observed when gPr80-defective virus was inoculated on immortalized mouse embryonic fibroblasts (MEF) and JTAg cells expressing the ecotropic receptor (Fig. 4B). The extent of the glycogag requirement for MLV-E infectivity therefore depends on the target cell type.

Because glycogag can substitute Nef for HIV-1, the ability of Nef to replace glycogag for MLV was tested. Nef expressed in trans in producer cells failed to rescue the infectivity of MLV lacking glycogag (Fig. 4C Left). The inability to rescue MLV infectivity was not due to a suboptimal Nef activity, because its expression could fully rescue the infectivity of HIV-1^Nef^- (Fig. 4C Right) and therefore might indicate mechanistic differences between gPr80 and Nef.

**Activities of gPr80 and Nef on Infectivity Have Similar Requirements and Properties.** Having established that gPr80 has a Nef-like activity on HIV-1, the similarity between the two proteins was further investigated.

The nature of the envelope glycoprotein can determine the requirements of gPr80 (Fig. 4A and B) and Nef (28, 29). Their activity on MLV and HIV-1 was therefore tested side-by-side on differently pseudotyped particles. Glycogag was strongly required for infectivity of MLV/HIV-1 pseudotypes inoculated onto NP2-CX4/CXCR4 cells but was dispensable for the infectivity of MLV pseudotyped with VSV-G (MLV/VSv; Fig. 5A Left), recapitulating the Nef requirement on HIV-1. On the other hand, HIV-1 particles pseudotyped with MLV-A or MLV-X Env glycoproteins were responsive to Nef (Fig. 5A Right), which, however, was no longer required for HIV-1 pseudotyped with MLV-E inoculated onto HT1080-mCat1. This evidence shows that Env glycoproteins, which render MLV dependent on Nef, are dispensable for the infectivity of gPr80, make HIV-1 responsive to Nef. The requirement of both proteins is therefore similarly determined by Env.

The cell type dependence of the activities of gPr80 and Nef on MLV-X and HIV-1 were also compared by using a panel of producer cell lines, which include adherent cell types (Fig. 5B). The requirements of Nef and gPr80 were both high for virions derived from Jurkat and CEM, moderate for viruses produced from 293T and A549, and low for viruses derived from HT1080 and TE671 cells. The requirement of both proteins is therefore similarly determined by the producer cell type. In addition, the absolute infectivity of wt virions generated by the different producer cell lines is variable (Fig. S2), suggesting that, in addition to gPr80 and Nef, cellular factors are important to modulate the infectivity of MLV and HIV-1.

Finally, to verify whether Nef and gPr80 are required at a similar stage of the virus life cycle, their effect on progression of reverse transcription in infected cells was compared (Fig. 5C). As detected by quantitative real-time PCR, HIV-1^Nef^- and MLV-X^gg^- produced significantly less late RT products than HIV-1^WT^ and MLV-X^gg^-; gPr80 had a similar effect on the accumulation of HIV-1 RT products (Fig. S3). The low abundance of RT products generated by
mutant viruses mirrors their defective infectivity (Fig. S3) and indicates that, like Nef, the activity of gPr80 on MLV and HIV-1 is also manifest during an early step of the infection process.

Discussion

Although the presence of glycosylated gag in gammaretroviruses was found >30 years ago, its function in the context of the retrovirus life cycle has remained enigmatic. In this study, the fortuitous presence of gammaretroviruses in HIV-1 producer cells revealed the ability of glycogag to rescue the infectivity of HIV-1\(\text{Net-}\) particles. gPr80 was then found crucial for MLV infectivity. The positive effect of gPr80 on gammaretrovirus replication in vivo and in some cases in vitro has been reported (41–47). Data presented here describe, in addition, the ability of glycogag to increase the intrinsic infectivity of retrovirus particles, an activity that shares striking functional similarity with Nef.

Being gPr80, a type II transmembrane protein, the conventional Gag residues are extracellular. However, in this study, the extracellular domain of gPr80 was found to be not strictly required for the activity on infectivity (Fig. 5C), indicating that the Gag residues and, therefore, the glycosylation status of gPr80, is not essential for the infectivity function.

Besides the evidence that glycogag can replace the activity of Nef for the infectivity of HIV-1, a functional similarity between the two proteins is supported by other observations.

(i) The requirement for gPr80 or Nef is similarly determined by the envelope glycoprotein (Fig. S4). Virions pseudotyped with envelope proteins, such as VSV-G, which require endosomal uptake, could bypass a block which targets Nef- and gPr80-defective virions that fuse directly at the cell membrane (28, 29). Interestingly, the entry of MLV-E has been described to occur via endocytic vesicles in a cell-type dependent manner (52, 53), a property which might explain the target cell-dependent variability of the gPr80 activity observed on MLV-E and the lack of Nef requirement for HIV-1(MLV-E) infecting HT1080 cells.

(ii) The requirement for gPr80 or Nef is similarly determined by the producer cell type (Fig. 5B). Both proteins could counteract a cellular condition that equally impairs both HIV-1 and MLV particles and which is differentially present in different cell types. Interestingly, the requirement of both proteins is strongest using producer lymphoid cells. Given the lymphotropic nature of HIV and MLV, the need for Nef or gPr80 activities could be a prerogative of lymphotropic viruses. Of note, consistent with this hypothesis, p12 of HTLV-I, another lymphotropic retrovirus, was also reported to have a Nef-like activity on HIV-1 infectivity (54). Incidentally, the evidence that infectivity of wt MLV and wt HIV-1 varies significantly with different producer cell lines (Fig. 1B and Fig. S2) suggests that additional cell-type specific activities operate to modulate retrovirus infectivity.

(iii) Glycogag and Nef have an identical intracellular distribution (Fig. S4). Despite the absence of sequence conservation, the two proteins have therefore similarly developed the ability to target the same cellular compartments, likely to be the TGN and coated pits (55, 56).

(iv) MLV\(\text{Net}\) and HIV-1\(\text{Net}\) are both defective at an early stage of the infection process (Fig. 5C), which precedes the completion of reverse transcription. Although in the case of HIV-1 a role of Nef during entry into the target cell was ruled out (26, 27, 57), this remains to be established for gPr80. However, glycogag does not alter association of Env with either MLV or HIV-1 particles (Fig. S5). Importantly, the effect of gPr80 on HIV-1 is also manifest during reverse transcription (Fig. S3), further indicating that its activity on HIV-1 resembles that of Nef.

Nef was unable to rescue the infectivity of MLV\(\text{Net}\) (Fig. 4C), whereas glycogag not only rescues HIV-1\(\text{Net}\)-, but can restore it to higher levels than HIV-1\(\text{Net}\) (Fig. 2B). This evidence could reflect a mechanistic difference. One possibility is that Nef requires the specific interaction with another lentiviral component to which glycogag cannot interact. Because Nef and glycogag are known to be incorporated into virions (58, 59), another possibility is that Nef cannot function as an MLV virion protein. However, abundant Nef was found in MLV particles (Fig. S5) (58), contrasting with its lack of activity on the gammaretrovirus and suggesting that its incorporation into virion is not sufficient for the effect on infectivity.

Interestingly, gPr80 was reported to affect the release of both MLV and HIV-1 by facilitating budding from lipid rafts (48). Intriguingly, Nef has also been reported to favor budding from specialized lipid domains (60–62). Whether this feature of gPr80 is linked to the activity on infectivity described here remains to be established, because a positive effect of gPr80 on MoMLV or HIV-1 release could not be observed in this study.

Nef performs several prominent activities, which include the ability to down-regulate cell surface receptors and to activate cellular kinases. However, Glycogag is unable to down-regulate the HIV-1 receptor CD4, the MLV-E receptor mCat-1 (Fig. S6), and MHC-I complexes (Fig. S6). The effect of glycogag on kinase activation remains to be experimentally assessed. However, those motifs present in Nef that are known to be important for recruiting and activating cellular kinases (13, 63) are not evident within the cytoplasmic tail of glycogag. The similarity between
the two proteins could therefore be limited to the activity on infectivity. Despite this limitation, the crucial role of gp80 for disease progression in infected animals (43, 45–47), the strong selective pressure that ensures its expression during infection in vivo (43, 45, 64), and its requirement for sustained virus replication in animals (41–44) are additional features that highlight a similarity with the Nef function.

In conclusion, a Nef-like requirement for retroviral infectivity is not unique to primate lentiviruses, as shown by the strong functional similarity between Nef and gp80. Intriguingly, the ORFs of the two proteins have evolved from unrelated genetic regions of divergent retroviruses and do not share any significant homology. The activity on infectivity of Nef and glycopag could therefore be the result of convergent evolution, highlighting a fundamental role in retrovirus biology.

**Materials and Methods**

**Plasmids.** Env- and Nef-defective HIV-1 NL4-3 provirus constructs have been described (32). The MLV provirus construct (MoMLV; accession no. J02255) and its derivatives are based on pNCA (65). Env-defective MLV was generated by introducing a frame-shift at a BspEI site in env. A replication-competent MLV-X was generated by replacing MoMLVEnv in pNCA with that of NZB-9:1. Expression vector for HIV-1vag, Env, HIV-1vag, EnvΔCT (that encodes a truncated HIV-1 Env that lacks 144 residues of the cytoplasmic domain, used to pseudotype MLV particles), and HIV-1vag were described (66). Env from MLV-NZB-9:1 was expressed from a pCDNA-based vector. Vectors expressing Amphotropic (4070-A), ecotropic (MoMLV) Env, and the vesicular stomatitis virus G protein (pMD.G) have been described (67, 68). Truncations of gPr80 with an N-terminal HA-Tag were cloned into the expression vector PEJ5. Stable cell lines expressing the ecotropic MLV receptor (mCAT-1) were generated by transduction with a pBABE-puro-based vector expressing mCAT-1, followed by puromycin selection.

**Virus Production and Infectivity.** Virions capable of a single round of replication were produced by transfecting suspension-growing cells with electroporation and adherent cells with FGENE-6 (Roche), using env-deficient HIV or MLV proviral DNA and vectors encoding viral Env glycoproteins at a 4:1 ratio. Cotransfections of HIV-1 adherent cells with FuGENE-6 (Roche), using


Supporting Information

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SI Text

VDJ Junction Sequence of the tdcr of JJu6 and JTAg Is Identical. DNA was extracted from JM, JJu6, and CEM cells. A PCR to amplify a TCR gene region encompassing the VDJ junction was performed by using primers 5′-AGTCGGTGACCCAGCTTGGC-3′ and 5′-ATCTTGGAAGCACTGCTGTTCGG-3′. A PCR product was only obtained from DNA derived from JTAg and JJu6. The DNA sequence of the PCR products was determined and found to be 100% identical in the two cell lines and to the sequence of the Jurkat T cell receptor active α-chain precursor mRNA (accession no. M12423).

Effect of gPr80 and Nef on Cell Surface Expression Levels of CD4 and MHC-I. JTAg cells and mouse immortalized fibroblasts MC57 were transfected with vectors encoding HA-gg189 or Nef-HA in combination with a plasmid encoding EGFP. Forty-eight hours after transfection, surface human CD4, and human and mouse MHC-I expression levels on cells gated for GFP expression were determined by two-color flow cytometry after staining with mouse anti-CD4, anti-human HLA-ABC, and anti-mouse H-2Kb (BD Pharmingen) followed by APC-conjugated anti-mouse (Jackson ImmunoResearch).

Virus Binding Assay. Virus binding assay for detection of mCAT-1 surface expression levels was performed on JTAg cells stably expressing mCAT-1 or control parental JTAg cells. Cells were transfected with vectors encoding HA-gg189 or Nef-HA in combination with a plasmid encoding EGFP. Forty-eight hours after transfection, cells were incubated with a MoMLV suspension produced from 293T cells transfected with pNCA at 4 °C for 60 min. Virus–cell complexes were stained with anti-RLV SU (Quality Biotech) followed by APC-conjugated anti-goat, and cells gated for GFP expression were analyzed.

Fig. S1. gPr80 is required for optimal infectivity of MLV. MLV-X restricted to a single cycle of replication produced from JTAg cells and normalized for RT activity was inoculated onto HT10180 cells. The figure shows immunofluorescence staining of infected target cells performed 48 h after infection by using an anti-RLV P30 antibody followed by Alexa-488 conjugated anti-goat antibodies.
Fig. S2. Absolute infectivity of HIV-1 and MLV-X derived from different producer cell types. Results expressed as percentage of WT in Fig. 5 are here shown as absolute infectivity values.

Fig. S3. gPr80 affects progression of HIV-1 reverse transcription. (A) gPr80 affects the steady-state levels of Nef-negative HIV-1 full-length viral cDNA, resembling the effect of Nef (Fig. 5C). (B–D) Relative infectivity of HIV-1 and MLV-X used in Fig. 5C. Viruses produced in JTAg cells and used in Fig. 5C and in A were serially diluted and inoculated onto HT1080 (B) or NP2-CD4/CXCR4 (C and D) respectively. Infected target cells were stained with anti RLV P30 anti HIV-1 p55/p2 and followed by Alexa-488 conjugated antibodies and quantified by scoring clusters of infected cells by fluorescence microscopy.
Fig. S4. Glycogag and Nef colocalize. Fluorescence microscopy shows colocalization of HA-gg189 with Nef-GFP but not with untagged GFP in Cos-7 cells (A) and in Jurkat TAg cells (B). Cells are cotransfected with vectors encoding HA-gg189 and Nef-GFP or GFP. Colocalization was observed in every cell found to coexpress HA-gg189 and Nef-GFP.

Fig. S5. Nef-HA and HA-gg189 are efficiently incorporated into MLV and HIV-1 particles. Neither Nef nor glycogag affect the incorporation of Env on HIV-1 and MLV. Env defective MLV and HIV-1 NLA-3 provirus constructs were transfected into TAg cells together with Env-expressing plasmids and vectors encoding Nef-HA or HA-gg189 as indicated. Virus supernatants were harvested 48 h after transfection and pelleted through a 25% sucrose cushion. Virus pellets and producer cell lysates were analyzed by SDS/PAGE followed by Western blotting using mouse anti-HA (HA.11; Covance), anti-HIV-1 p55/p2 and anti-HIV-1 gp120 ARP423 (National Biological Standards Board), anti-RLV P30 (Quality Biotech) and anti-β-actin (Sigma). No virus controls were obtained by replacing provirus constructs with empty vectors to provide evidence that Nef-HA and HA-gg189 are genuinely detected in virus particles.
Unlike Nef, glyco gag does not down-regulate retrovirus receptors and MHC-I. (A) gPr80 does not affect cell surface expression levels of human CD4 and MHC-I in JTAg cells. (B) Neither gPr80 nor Nef affect surface levels of mouse MHC-I (H-2K^b) in MC57 mouse fibroblasts. Of note, the failure of HIV-1 Nef to down-regulate MHC-I in mouse cells has been reported (1). (C) Virus binding assay for detection of cell surface mCAT-1 on JTAg/mCat-1 cells (SI Text). (Left) The presence of mCat-1 on JTAg cells promotes virus binding detected by using an anti-SU antibody. (Center and Right) Nef and gPr80 expression in JTAg/mCat1 do not affect efficiency of SU binding and, therefore, of mCat-1 surface expression level.

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