Journal of Virological Methods 171 (2011) 299–302

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Replication of biotinylated human immunodeficiency viruses

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Article history:
Received 30 June 2010
Received in revised form 12 October 2010
Accepted 8 November 2010
Available online 16 November 2010

Keywords:
Human immunodeficiency virus type 1 and type 2
Biotinylation
Matrix
Integrase
vpx
vpr

A B S T R A C T

Previous work demonstrated recently the adaptation of the *Escherichia coli* biotin ligase BirA – biotin acceptor sequence (BAS) labeling system to produce human immunodeficiency virus type 1 viruses with biotinylated integrase ($NLXIN_B$) and matrix ($NLXMA_B$) proteins (Belshan et al., 2009). This report describes the construction of an HIV permissive cell line stably expressing BirA (SupT1.BirA). Consistent with the results in the previous report, $NLXMA_B$ replicated similar to wild-type levels and expressed biotinylated Gag and MA proteins in the SupT1.BirA cells, whereas the replication of $NLXIN_B$ was reduced severely. Three additional HIV type 2 (HIV-2) viruses were constructed with the BAS inserted into the *vpx* and *vpr* accessory genes. Two BAS insertions were made into the C-terminal half of the Vpx, including one internal insertion, and one at the N-terminus of Vpr. All three viruses were replication competent in the SupT1.BirA cells and their target proteins biotinylated efficiently and incorporated into virions. These results demonstrate the potential utility of the biotinylation system to label and capture HIV protein complexes in the context of replicating virus.

Human immunodeficiency virus 1 (HIV-1), a member of the genus *Lentivirus* in the *Retroviridae* family, interacts with a myriad of cellular proteins to complete a productive replication cycle. Identifying and characterizing these interactions may lead to an increased knowledge of HIV pathology and the eventual discovery of new targets for therapeutic inhibition of HIV replication. Three recent genome-wide siRNA screens individually identified >200 potential factors critical for the productive infection by the virus (Brass et al., 2008; Konig et al., 2008; Zhou et al., 2008). Unexpectedly the overlap between these screens was minimal (~16 genes). Moreover, the validation of all the potential targets will take years to complete. An alternative method to genome wide screening for viral factors is the characterization of HIV-host cell proteome. Whole cell, or so-called "shotgun" proteomics, has proven to be of limited utility given current mass spectrometry (MS) technologies. Historically the analyses of protein–protein interactions have been limited to screens performed with individual viral proteins. A common protein-based purification strategy that has been utilized to identify potential HIV-interacting cellular proteins is to capture protein complexes using single or tandem-affinity epitope tagged viral proteins to capture protein complexes. This approach is not ideal since the viral bait protein is usually expressed as a standalone expression construct, and the experiments are performed in cell lines that are transfected efficiently, but not necessarily permissive for HIV-1 infection. Genetic studies have established that HIV infection results in a rearrangement of the expression of a multitude of genes and gene pathways (van't Wout et al., 2003). Therefore it would be advantageous to study HIV-cellular interactions in the context of productive HIV infection. This requires the insertion of an affinity tag into a HIV-1 protein that maintains protein function and virus replication. The effectiveness of this strategy was demonstrated by the identification of the interaction of HIV-1 Vif with Cul5 using a molecular clone containing HA-tagged Vif (Yu et al., 2003).

A previous study reported the adaptation of the *Escherichia coli* biotin ligase BirA – biotin acceptor sequence (BAS) labeling system to biotinylate HIV-1 in vivo (Belshan et al., 2009). For this system the 20 amino acid BAS is inserted into the bait protein. Co-expression of BirA with a BAS-containing protein results in the covalent attachment of biotin to a central lysine residue in the BAS (Beckett et al., 1999; Schatz, 1993). Two HIV-1 molecular clones were characterized previously that contained BAS insertions into the C-terminus of the matrix ($NLXMA_B$) and integrase ($NLXIN_B$) proteins (Belshan et al., 2009). Both viruses produced virions containing their respective biotinylated proteins from 293T cells expressing BirA. Replication assay in T-cells demonstrated that the MA insertion was well tolerated, but the IN insertion reduced virus viability. Single-round infectivity assays with biotinylated viruses confirmed that the MA virus was as fit as wild-type virus, but the biotinylated IN virus was not infectious. The construction of a 293T cell line that exhibited stable expression BirA for the

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doi:10.1016/j.jviromet.2010.11.005

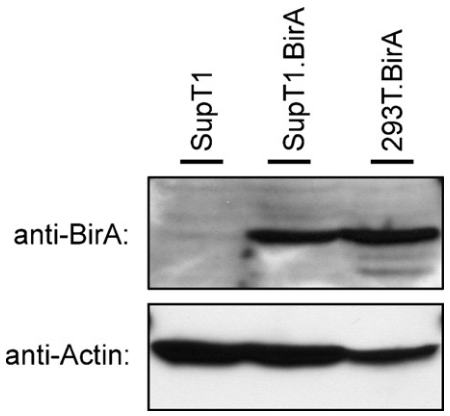

Fig. 1. Cell lines that stably express BirA. Western blot analysis of BirA expression in SupT1, SupT1.BirA, and the previously described 293T.BirA cell lines (top panel). Cells were lysed with M-PER solution (Pierce Biotechnology, Rockford, IL, USA), the lysates clarified by centrifugation and separated by SDS-PAGE. BirA was detected by Western blot using an anti-BirA antibody (Genway Biotech, San Diego, CA, USA) followed by an anti-chicken IgY-HRP conjugated secondary antibody. A control Western blot for Actin (Santa Cruz Biotechnology, Santa Cruz CA USA) was also performed as a normalization control (lower panel).

consistent production of biotinylated virus was also reported. A recent report also described the production of biotinylated IN and MA using a lentiviral transduction system (Benkhelifa-Ziyyat et al., 2010); however that system does not permit productive replication of biotinylated proteins.

Belshan et al. (2009) demonstrated the feasibility and specificity of the system to biotinylate HIV proteins in vivo, however the labeling was limited to virus produced by 293T cells expressing BirA. The next goal of these studies was to label HIV proteins in the context of replicating virus. To accomplish this, a HIV-1 permissive cell line that expressed stable levels of BirA was produced. 5×10^7 SupT1 cells were electroporated with 20 μg of the pc6BirA expression plasmid in a 0.4 cm gap electoporation cuvette and electroporated using a Gene Pulser II electroporator (Bio-Rad Laboratories, Hercules, CA USA) set to 300 V and 975 μF. After electroporation the cells were expanded for 2 days and then passaged into media supplemented with 10 μg/ml Blasticidin S to select for pc6BirA plasmid maintenance. Cells were propagated, and media changed every 2–3 days, until visible cell growth was observed by media color change (approximately 3 weeks). Single cell clones were obtained by seeding the cells into 96-well plates at 0.1 cells/well. Cells were grown until colonies were visible and only wells with single colonies were expanded. Once expanded sufficiently the cell clones were examined for BirA expression by Western blot. Several SupT1.BirA cell lines were identified to express BirA at high levels and a single high expressing cell line was chosen for future experiments. The expression of BirA in the SupT1.BirA cell line as well as the 293T.BirA cell line described previously is demonstrated in Fig. 1.

To establish that the SupT1.BirA cells retained the ability to support HIV replication and test their ability to biotinylate HIV proteins in the context of ongoing replication, the cells were infected with wild-type HIV-1 NLX and the $NLXMA_B$ and $NLXIN_B$ viruses and supernatants were collected (Fig. 2A). SupT1.BirA cells were inoculated overnight with normalized amounts of each virus, the virus removed the next day, and the cells propagated for 11 days. The supernatants were sampled in triplicate at various days post infection and virus replication measured by an in vitro [^{32}P]TTP incorporation reverse transcriptase (RT) assay (Belshan et al., 2009). The parental NLX virus replicated well in the SupT1.BirA cells indicating that the expression of BirA was not deleterious for HIV replication. The replication of the $NLXMA_B$ virus was similar to wild-type NLX virus, consistent with the previous findings

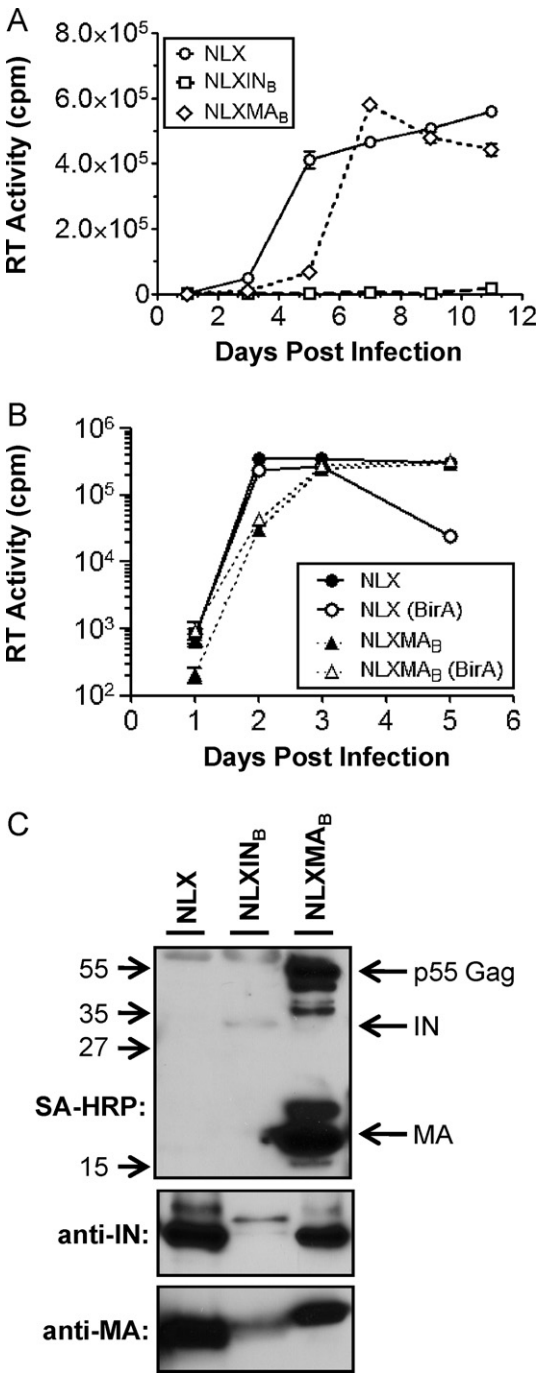

Fig. 2. HIV-1 replication and biotinylation in SupT1.BirA cells. (A) SupT1.BirA cells were inoculated overnight with normalized levels of the indicated viruses, the cells washed, and propagated. Supernatants were collected in triplicate and clarified by centrifugation on the days indicated. At the end of the experiment the samples were analyzed for exogenous RT activity using a [^{32}P]TTP incorporation assay. (B) SupT1 (black symbols) and SupT1.BirA cells (open symbols (BirA)) were infected in parallel with normalized amounts of the indicated virus and monitored for replication as described in (A). Error bars in both (A) and (B) denote the standard deviation for the triplicate samples in one experiment. (C) At the end of replication experiments 12 ml of supernatant was collected, clarified, and concentrated by ultracentrifugation through a 20% sucrose cushion (w/v in PBS). Samples were resuspended in 1× Sample Buffer, separated by SDS-PAGE and the indicated proteins detected by Western blot. The location of viral proteins in the streptavidin-horseradish peroxidase (SA-HRP) blot is shown at the right of the blot and the approximate location of molecular weight markers is shown on the left. The data shown in (A) and (C) is representative of three independent experiments and (B) is representative of duplicate experiments.