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Differential interaction between human and murine Crm1 and lentiviral Rev proteins

Yan Yue, Ayse K. Coskun, Navneet Jawanda, Jim Auer, Richard E. Sutton*

Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520, United States

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ABSTRACT

Mice have multiple obstacles to HIV replication, including a block of unspliced and partially spliced viral mRNA nuclear export. In human, Rev binds to the Rev-response element and human (h) Crm1, facilitating nuclear export of RRE-containing viral RNAs. Murine (m) Crm1 is less functional than hCrm1 in this regard. Here we demonstrated that in biochemical experiments mCrm1 failed to interact with HIV Rev whereas hCrm1 did. In genetic experiments in human cells, we observed a modest but significant differential effect between mCrm1 and hCrm1, which was also true of other lentiviral Revs tested. Triple mutant hCrm1 P411T-M412V-F414S behaved similarly to mCrm1, whereas mCrm1 with T411P-V412M-S414F regained some activity, although contribution of additional residues to its function can not be excluded. Similar results were observed in murine cells. This suggests a differential interaction between hCrm1 and mCrm1 and many lentiviral Revs, which may partially explain the HIV replicative defect in mice.

1. Introduction

Since the recognition of AIDS in 1981 more than thirty-five years ago, nearly 70 million individuals have been infected with human immunodeficiency virus type 1 (HIV) and roughly half have died (http://www.unaids.org/en/resources/documents/2016/AIDS-by-the-numbers). Although the introduction of HAART two decades ago has been truly transformative, HIV disease, once invariably fatal, remains incurable. The development and testing of a safe and efficacious prophylactic HIV vaccine, a long-sought goal, has been stymied by the absence of a small animal model. Although chimpanzees can be infected with HIV there is now a moratorium on their experimentation (Kaiser, 2015), and the use of simian-HIV (SHIV) hybrids in rhesus macaques is limited by animal availability and expense. Ideally, a fully permissive small animal model would allow higher throughput testing of candidate vaccines and correlates of immunity, if any. Unfortunately, current humanized mouse models, although highly sophisticated and informative (Denton and Garcia, 2011), do not allow for vaccine testing.

Most rodent species, including the mouse, have multiple blocks to HIV replication, including at the level of viral entry and transcriptional elongation. Even when those obstacles are circumvented using entry factors and human cyclin T1, which allows for high level viral RNA production, very little infectious HIV is produced from murine cells (Coskun et al., 2007, 2006; Elinav et al., 2012; Sherer et al., 2011; Swanson et al., 2010). We and others have pinpointed a major post-integration block in mouse cells at the level of unspliced and partially spliced viral RNA nuclear export (Elinav et al., 2012; Sherer et al., 2011; Swanson et al., 2010).

All retroviruses require nuclear export of intron-containing mRNA for productive, high level replication, and for the lentiviruses this process requires a cis-acting RNA sequence and trans-acting viral and cellular proteins. HIV Rev is an essential regulatory protein that is highly conserved among all viral isolates and clades (Malim et al., 1989a, b, c). It is encoded by a fully spliced mRNA, and is in a different reading frame than Tat, but shares precisely the same major intron. After cytosolic translation, Rev is imported into the nucleus, where it multimerizes on the Rev-response element (RRE, present within that same intron) to allow nuclear export of unspliced and partially spliced viral mRNAs, including genomic RNA (Madore et al., 1994; Malim and Cullen, 1991; Mann et al., 1994; Szilvay et al., 1997; Vercruysse and Daelemans, 2013). Based upon biochemical analysis it is thought that 6–8 Revs cooperatively bind a single RRE (Cook et al., 1991; Daly et al., 1989; Daugherty et al., 2008; Daugherty et al., 2010; Heaphy et al., 1991; Holland et al., 1990; Wingfield et al., 1991); a low resolution structure has demonstrated a Rev dimer binding across a bent RRE (Fang et al., 2013). In the nucleus Rev-RRE complex interacts with host factors chromosome region maintenance 1 (Crm1) and Ran-GTP, and in the cytosol the complex dissociates to be recycled to the nucleus to export additional cargo, leaving RRE-containing viral mRNA in the cytosol. In the absence of Rev multiple viral proteins cannot be

* Corresponding author.
E-mail address: richard.sutton@yale.edu (R.E. Sutton).

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synthesized, including Gag, Pol, and Env, and genomic RNA is trapped in the nucleus or spliced. Interestingly there are no cellular homologs of Rev. Only rarely are intron-containing cellular mRNAs exported to the cytosol via an analogous system (Li et al., 2006; Wang et al., 2015), whereas all lentiviruses routinely use the Rev-RRE complex for trafficking of viral mRNAs with retained introns.

We had previously observed a decrease in unspliced, intron-containing HIV mRNA in infected murine cells (Coskun et al., 2007, 2006; Elinav et al., 2012). The presence of human chromosome 2 in murine cells largely reversed this defect and significantly enhanced infectious virus production (Coskun et al., 2006). We and other investigators identified human (h)Crm1 as the likely gene product on chromosome 2 responsible for this effect (Elinav et al., 2012; Nagai-Fukataki et al., 2011; Okada et al., 2009; Sherer et al., 2011). Expression of hCrm1 in murine cells allowed export of intron-containing HIV mRNAs from the nucleus and significantly boosted virus production, whereas murine (m) Crm1 was non-functional. In our hands this defect mapped to HEAT (Huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast kinase TOR1) repeat 9A of hCrm1, specifically to amino acid residues 411, 412, and 414 (Elinav et al., 2012). The effect of hCrm1 was even more pronounced using feline immunodeficiency virus, and hCrm1 acted more than additively with hSRp40, a serine-arginine rich splicing factor, to increase infectious virus production from mouse cells. More recently it was demonstrated that the addition of a second nuclear export signal to HIV Rev allowed for both enhanced HIV Capsid (CA) production and infectious virus release from murine cells (Aligeti et al., 2014), consistent with a fundamental defect in nuclear export of viral mRNAs in rodent cells.

The question remained as to why hCrm1 was functional and mCrm1 was not in terms of Rev and HIV mRNA nuclear export, and previously we proposed three possibilities (Elinav et al., 2012). One was that human cells had a positively acting factor that somehow stabilized the hCrm1 and Rev-RRE complex (but did not do so for mCrm1); a second was that murine cells had a negatively acting factor that disrupted the mCrm1 and Rev-RRE complex (but did not do so for hCrm1). Lastly, we hypothesized that hCrm1 simply interacted more favorably or strongly with Rev-RRE complex, compared to mCrm1. We decided to test the third model by evaluating hCrm1 and mCrm1 interaction with lentiviral Rev proteins, first biochemically and then genetically, the latter using a mammalian two-hybrid system in both human and murine cells.

2. Results

To test whether there is a differential interaction between hCrm1 and mCrm1 and Rev-RRE complex, we first turned to a biochemical method initially developed by Cullen and colleagues (Bogerd et al., 1998), using purified, bacterially expressed HIV Rev-GST and Ran-GTP proteins, along with in vitro transcribed HIV RRE RNA and in vitro translated hCrm1 or mCrm1 proteins. Both Rev-GST and RevM10-GST fusions were purified using glutathione beads as ~45 kD proteins (Fig. 1). When incubated with Ran-GTP and either in vitro translated, HA epitope-tagged hCrm1or mCrm1, only HA-hCrm1 was precipitated, but only in the presence of HIV Rev-GST fusion and RRE RNA (Fig. 1). This is consistent with prior work showing ~2× stronger binding of purified hCrm1 compared to mCrm1 to HIV-1 Rev (Booth et al., 2014). As expected, RevM10-GST fusion failed to precipitate or bind either HA-Crm1 protein, in the presence or absence of RRE.

To confirm and extend those findings of a differential interaction between hCrm1 and mCrm1 and Rev-RRE, we first attempted to use the yeast two and three hybrid systems, but failed to observe a detectable interaction between any of the Crm1s and Rev, in the presence or absence of HIV RRE RNA. We then turned to the luciferase complementation system in which protein interaction between amino and carboxy terminal firefly luciferase (FFLUC) fusion proteins results in detectable FFLUC activity by relative light units (RLUs) (Luker et al., 2004). Using this system we were able to detect a strong HIV Rev-Rev

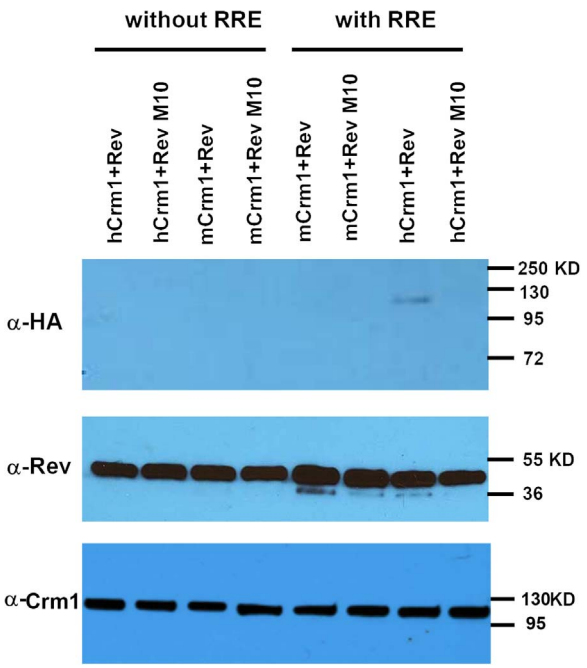

Fig. 1. In vitro interaction of human or mouse Crm1 with HIV Rev. Top: GST pull-down assay was performed to compare binding of human vs. mouse Crm1 with HIV1 Rev/RevM10-GST in the presence of RanQ69L-GTP, with or without HIV1 RRE. Middle: input for each reaction probed with anti-Rev antibody; bottom: input for each reaction probed with anti-Crm1 antibody. Molecular mass markers are shown to the right of each panel. This experiment was performed once.

genetic interaction but not between Rev and any Crm1 fusion protein. Because the luciferase complementation system may only allow readout when each fusion partner is small enough to permit enzymatic catalysis and full-length Crm1 is greater than 100 kD in size, we next attempted the mammalian two-hybrid system, knowing that we could also separately transfect in HIV RRE RNA in plasmid form, driven by either RNAPII or III promoters.

In the mammalian two-hybrid system one protein fusion is with the Gal4 DNA binding domain (DBD) and the other is with the herpes simplex virus VP16 activation domain (AD), with positive control fusions utilizing cellular p53 and SV40 TAg as known, strong interactors. The reporter we used had five repeats of the 17-mer Gal4 DNA binding motif driving FFLUC. Initially we tested a series of Rev and Crm1 fusion proteins in 293T transient transfection assays to identify a combination that gave the lowest level of autoactivation. Although the Gal4DBD-Crm1 fusions gave some FFLUC readout when transfected in the absence of any VP16 fusion partner, the autoactivation was much lower compared to that of the VP16AD-Crm1 fusions (not shown).

Based on these results, we tested the functionality of the Gal4DBD-Crm1 fusions in a functional assay, transfecting them into murine B78 cells that had an integrated HIV reporter vector encoding both a truncated form of human cyclin T1 and blasticidin resistance (*bsd*) (Coskun et al., 2007), along with VSV G expression construct and an HIV vector encoding a truncated form of human cyclin T1 and eYFP. The B78 cells express murine Crm1, and this assay allows us to test the functionality of other Crm1 constructs (Fig. 2A). Human cyclin T1 is required since expression of both eYFP and *bsd* in the vector used are dependent upon the HIV long terminal repeat and the presence of Tat. As anticipated, the non-fused, full-length hCrm1 had the greatest activity in terms of infectious virus release, as measured by the number of blasticidin-resistant colonies on HOS cell targets (Fig. 2B). The Gal4DBD-hCrm1 fusion had ~25% of the activity of the non-fused hCrm1 but was significantly more active than the Gal4DBD-mCrm1 fusion and the Gal4DBD-hCrm1 411–412-414 mutant. As reported previously (Aligeti et al., 2014), the 2xNES-Rev-mCherry expression plasmid gave roughly