TRIM28 mediates primer binding site-targeted silencing of Lys1,2 tRNA-utilizing retroviruses in embryonic cells

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Murine leukemia viruses (MLVs) and related retroelements are potentially restricted in embryonic cells by postintegration transcriptional silencing, likely to protect the germ line from insertional mutagenesis. This silencing is in large part attributable to the presence of a nuclear repression complex, which targets a sequence element of the proviral DNA, the repressor-binding site. The repressor-binding site closely overlaps the tRNA primer binding site, a highly conserved sequence essential for virus replication and defining the site of initiation of DNA synthesis during reverse transcription. We have recently demonstrated that the cellular corepressor TRIM28 is recruited to the proline tRNA primer-binding site used by many MLVs and is required to mediate this silencing. Here, we show that TRIM28 is also required for the restriction of retroviruses using a completely distinct tRNA for the priming of their DNA synthesis, namely Lys-1,2 tRNA. These results generalize the role of TRIM28 in retroviral restriction and suggest that this system has evolved to restrict multiple retroviruses.

HP1 | KAP-1 | MLV | PBS

Murine leukemia viruses (MLVs) are unable to replicate in embryonic cells (1–3). The block to replication occurs after the viral DNA has integrated into the host genome and is caused by transcriptional silencing of the proviruses (2, 4). This silencing is the result of several cumulative effects that include poor enhancer function of the 5' LTR (5–7), the binding of several cell-type specific transacting transcriptional repressors (6, 8), and *de novo* DNA methylation (4). A critical target of this repression is the DNA element known as the repressor binding site (RBS) (2, 5, 9, 10). The RBS element comprises 17 bp that overlap closely with the 18-bp primer binding site (PBS) of MLV (2), a sequence complementary to the cellular proline tRNA (tRNA^{Pro}) used to prime minus-strand DNA synthesis during reverse transcription (11). This silencing can be abrogated by a single base-pair substitution in the PBS known as the B2 mutation (2). Characterization of RBS-mediated restriction demonstrated that the 17-bp sequence was able to induce transcriptional silencing of reporter constructs in EC cells independently of position and orientation of the RBS sequence (10, 12). Furthermore, exonuclease III protection assay and EMSA experiments revealed the presence of a DNA-binding activity that is specifically enriched in stem cell nuclear extracts (10, 12). Based on these experiments, a trans-acting DNA-binding factor was postulated to exist; we have recently demonstrated that a high molecular weight complex binds the RBS sequence, and that a key component of this complex is the TRIM28 protein (13). TRIM28 (also known as Kap-1, or Tif1-beta) is a well characterized transcriptional corepressor that is recruited to its target genes by interactions with the Krüppel associated box (KRAB) zinc-finger DNA-binding proteins (14). This interaction is mediated via the KRAB box domain and leads to these DNA-binding proteins, serving as sequence-specific transcriptional repressors (15). TRIM28 functions as a transcriptional corepressor by, in turn, recruiting several other factors involved in transcriptional silencing and heterochromatin formation, including the NuRD histone deacetylase complex,

the histone H3 K9 methyltransferase ESET, and the heterochromatin-associated protein HP1 (16–18). As a component of the RBS-binding complex, TRIM28 is recruited to the PBS of MLV in EC cells, and functional TRIM28 is absolutely required for RBS-mediated restriction of MLV (13, 19). TRIM28 recruitment to integrated silenced proviruses in EC cells leads to the recruitment of HP1 γ (13), and disruption of the HP1-binding domain in TRIM28 leads to inactivation of the RBS-mediated silencing machinery (19). This suggests that recruitment of HP1 to proviruses is critical for their subsequent silencing.

Retroviruses, such as visna, spuma, and Mason-Pfizer monkey viruses, contain a distinct PBS sequence corresponding to tRNA^{Lys-1,2} (PBS^{Lys-1,2}) (20–22). MLV-based vectors using this sequence have been shown to be specifically restricted in embryonic cells (9, 23). Furthermore, it has been demonstrated that a single point mutation in the PBS^{Lys-1,2} sequence is able to relieve this restriction in a fashion similar to the B2 mutation in the PBS^{Pro} sequence (2, 23). These observations suggest that the PBS^{Lys-1,2} may recruit silencing machinery similar to the PBS^{Pro}, and thus might similarly involve TRIM28. We sought to investigate directly whether TRIM28 is also required for restriction of PBS^{Lys-1,2} sequences in embryonic cells and thus determine whether TRIM28-mediated silencing of PBS sequences is a more generalized mechanism of retroviral restriction.

Results

Previous work from many laboratories has documented the presence of strong sequence-specific DNA-binding activity for the PBS^{Pro} of MLV in lysates of embryonic cells (9, 12, 13, 24). Similar tests for DNA-binding activity in F9 EC cell extracts, when performed using the DNA sequence of PBS^{Lys-1,2}, the PBS used by retroviruses such as visna, spuma, and Mason-Pfizer monkey virus (20–22), yielded a complex detected by EMSA with a shift indicative of similar size as the complex that binds to the PBS^{Pro} sequence (9) (Fig. 1A). The PBS^{Lys-1,2} has also been shown to specifically induce the functional restriction of viral gene expression in EC cells (9, 23). We sought to determine if the complex binding to this PBS sequence also contained TRIM28. Nuclear extracts were prepared from the PCC4 EC cell line, as well as from a pool of cells that had been stably transduced with a retroviral vector expressing an anti-TRIM28 siRNA hairpin (PCC4 111), and an equivalent pool of PCC4 cells expressing a scrambled siRNA control (PCC4 SCRAM) (13). These nuclear extracts were then

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Fig. 1. A TRIM28 complex binds to the PBS^{Lys-1,2} sequence. (A) (Left) Nuclear extracts from PCC4 WT, PCC4 SCRAM, and PCC4 111 (TRIM28 knock down) cells were used in EMSA reactions with ³³P-labeled 28-bp probes PBS^{Pro} (PBS Pro) or PBS^{Lys-1,2} (PBS Lys-1,2). Samples were run on the same gel and intervening lanes were removed. + anti-TRIM28 denotes addition of 3 μg of anti-TRIM28 monoclonal antibody to EMSA reactions. Resultant supershift is marked with an asterisk. (Right) Western blots performed on nuclear extracts probed with anti-TRIM28 and anti-β-actin antibodies. (B) (Left) EMSA reactions performed as above except nuclear extracts were also prepared from PCC4 111 cells infected (moi 100) with either an adenovirus expressing TRIM28-myc/HIS (AdTRIM) or a control empty adenovirus vector (Ad). + anti-myc denotes addition of 1.6 μg of anti-myc monoclonal antibody to EMSA reactions. Resultant supershift is marked with a filled circle. (Right) Western blots performed on nuclear extracts probed with anti-myc, anti-TRIM28, and anti-β-actin antibodies.

used in EMSA reactions with a 28-bp probe containing either 17 bp of the PBS^{Pro} or of the PBS^{Lys-1,2}, as described (9). Nuclear extracts from PCC4 and PCC4 SCRAM cells both produced an EMSA shift of the PBS^{Lys-1,2} probe of approximately the same size as for the PBS^{Pro} probe (see Fig. 14). Unlike the PBS^{Pro} probe, several additional smaller shift complexes were also detected with the PBS^{Lys-1,2} sequence. The identity of these bands is unknown. In the absence of TRIM28 (PCC4 111), the major shifted species was no longer detected.

To confirm that TRIM28 is a component of this complex, an anti-TRIM28 antibody was added to each EMSA reaction and was able to specifically supershift the complex formed on the PBS^{Pro} sequence and the complex binding to the PBS^{Lys-1,2} sequence (see Fig. 1A). Upon knockdown of TRIM28 in PCC4 111 cells, the supershift band was not observed. The effectiveness of the knockdown was confirmed by assaying total cellular levels of TRIM28 by Western blot (see Fig. 1A). To demonstrate that specific depletion of TRIM28 from PCC4 cells by RNAi was responsible for the disappearance of the PBS^{Lys-1,2} RBS shift, an adenovirus was used to transiently re-express an RNAi-resistant TRIM28-myc/HIS construct in PCC4 111 cells. The transient expression of TRIM28-myc/HIS restored the RBS shift with the 28-bp PBS^{Lys-1,2} probe, and infection with an “empty” adenovirus vector not expressing TRIM28-Myc/HIS did not (Fig. 1B). To demonstrate that the RBS band shift seen was derived from the re-expressed tagged TRIM28-Myc/HIS construct, the same EMSA was performed in the presence of an anti-myc antibody; this antibody was able to supershift

this recapitulated myc tagged complex but did not shift the endogenous complex (see Fig. 1*B*). Western blots were performed to confirm the expression of TRIM28-myc/HIS in the adenovirus-infected lines (see Fig. 1*B*).

Because TRIM28 is not a DNA-binding protein, it likely depends on other sequence-specific DNA-binding proteins for its function. If it is recruited to different viral PBS sequences by two different DNA-binding proteins, then two corresponding different DNA probes should not cross-compete with each other. To test this notion, we performed EMSA reactions with either the PBS^{Lys-1,2} or PBS^{Pro} 28-bp probes in the presence of increasing quantities of either cold probe acting as a competitor (Fig. 2). As would be expected, PBS^{Pro} and PBS^{Lys-1,2} were both effectively competed by addition of unlabeled DNAs of their own sequence, even at low DNA concentrations (5 μ g/ml) (see Fig. 2). There was, however, no cross-competition between probes even at concentrations as high as 125 μ g/ml, an $\approx$ 500-fold excess of cold probe (see Fig. 2). This suggests strongly that these DNA sequences are indeed recognized by two separate DNA-binding activities.

A single point mutation in the PBS^{Lys-1,2} sequence is able to relieve the repression it induces in EC cells (23). This mutated PBS, named PBS^{Lys-1,2(m)}, would be predicted to be unable to bind TRIM28 if TRIM28 were also responsible for the restriction of the PBS^{Lys-1,2} sequence in EC cells. To test this hypothesis, EMSA reactions were performed with 28-bp PBS^{Lys-1,2} and PBS^{Lys-1,2(m)} probes (Fig. 3). As predicted, the PBS^{Lys-1,2(m)} probe

Fig. 2. The RBS complexes binding to PBS^{Pro} and PBS^{Lys-1,2} DNA sequences have different DNA-binding domains. Nuclear extracts from PCC4 WT cells used in EMSA reactions with ³³P-labeled 28-bp probes containing PBS^{Pro} (Pro) or PBS^{Lys-1,2} (Lys-1,2). Increasing amounts of cold probe were added as indicated (0.1, 0.5, and 2.5 μ g of cold DNA). Samples were run on same gel and intervening lanes removed.

did not show the TRIM28 complex RBS shift. To further investigate the relative affinities of the TRIM28 PBS^{Lys-1,2} binding complex, DNA competitions were performed with either WT PBS^{Lys-1,2} or PBS^{Lys-1,2(m)} cold probe (see Fig. 3). It was observed that the PBS^{Lys-1,2(m)} cold probe was not able to significantly compete for binding of the TRIM28 RBS complex, whereas the PBS^{Lys-1,2} probe did (see Fig. 3), demonstrating that the Lys-1,2(M) mutation has a considerably lower affinity for the TRIM28 RBS binding complex. These results are consistent with the fact that the mutation relieves restriction in EC cells.

Having determined that the TRIM28 binding to the PBS^{Lys-1,2} sequence correlated with its ability to cause repression in EC cells, we sought to determine whether knock-down of TRIM28 relieved this repression. An M-MLV based construct expressing the neomycin resistance gene from the 5'LTR was modified by replacing the PBS^{Pro} with the 17-bp corresponding to PBS^{Lys-1,2} (named

MLV-K2-NEO), which has previously been shown to be sufficient to induce repression in an Akv-MLV based vector (23). An identical vector with the 17-bp corresponding to PBS^{Lys-1,2(m)}, which should be resistant to this restriction, was also constructed (MLV-K2M-NEO).

It has been demonstrated that in the case of HIV-1, the tRNA used to prime reverse transcription can be influenced by sequences outside of the PBS (25), and that artificially introduced PBS sequences quickly revert back to the WT tRNA^{Lys-3} sequence (26). To ensure that these newly constructed M-MLV vectors were both using tRNA^{Lys-1,2} to prime reverse transcription, stocks of both viruses pseudotyped with the vesicular stomatitis virus (VSV) G envelope protein were used to infect Rat2 cells. The PBS sequences from both pools of infected cells were amplified by PCR and the products were sequenced. (Fig. 44). The DNA sequences confirmed that, as expected, the MLV-K2-NEO construct produced

Fig. 3. The binding affinity of the TRIM28-RBS complex is significantly lessened by the Lys-1,2(M) mutation. Nuclear extracts from PCC4 WT cells used in EMSA reactions with ³³P-labeled probes containing PBS^{Pro} (Pro), PBS^{Lys-1,2} (Lys-1,2), or PBS^{Lys-1,2(M)} [Lys-1,2(M)]. Increasing amounts of cold probe were added to reactions as indicated (10 ng, 50 ng, 250 ng, and 1 μg of cold DNA).

Fig. 4. TRIM28 is required to induce repression in MLV with a PBS^{Lys-1,2}. (A) DNA sequence electropherogram profiles of PCR products of PBS sequences amplified from pools of cells infected with VSV-G-pseudotyped M-MLV particles containing either the MLV-K2-NEO or MLV-K2M-NEO vector. (B) Rat2, PCC4, PCC4 SCRAM, or PCC4 111 cells were infected with VSV-G pseudotyped M-MLV particles containing either MLV-K2-NEO (K2) or MLV-K2M-NEO (K2M) constructs. Infection efficiency in each cell line was monitored by colony count after 2 weeks of G418 selection. Graph shows ratio of K2M/K2 infection efficiency in each cell line, normalized with Rat2 = 1. Error bars show $\pm$ standard error, with $n = 5$.

proviruses that solely contained the tRNA^{Lys-1,2} sequence. The MLV-K2M-NEO construct produced a mixture of integrants containing both the Lys-1,2(M) and Lys-1,2 sequence. This partial reversion, also as expected, takes place during reverse transcription, as one strand of the double-stranded viral DNA derives its sequence from the host tRNA and not the viral genome sequence (27). After integration of the provirus and cell division, without mismatch repair, half the cells will contain the WT PBS and half the mutant PBS. These results validate the expected structures of the reporter constructs.

We next asked whether the RBS-mediated repression manifest in PCC4 cells was attenuated in TRIM28-depleted cells. M-MLV particles pseudotyped by the VSV G protein and containing either the MLV-K2-NEO or the MLV-K2M-NEO vectors were produced, and the infectivity of each of these virus preparations was determined by colony formation assays in medium containing G418. The Lys-1,2 vector (K2) is repressed by RBS binding activity and the Lys-1,2(M) vector (K2M) is not (23), and therefore the ratio of the titers of these viruses in a particular cell line is a measure of the RBS activity in that cell line. Cells infected with K2M contain a mixture of unrepressible (M) and

repressible (WT) proviruses; the unrepressed mutant proviruses predominate in terms of their contribution to the expression in repressive cells. Because of the partial reversion, the true ratio of K2M/K2 repression should be approximately twice the observed ratio of titers of the virus preparations. However, here we simply report the observed ratio of titers as the simplest and adequate measure of repression of the fully WT PBS-containing over that of the (partially) mutant PBS-containing viruses.

Rat2 cells lack TRIM28-mediated repression activity, and therefore the MLV-K2-NEO and MLV-K2M-NEO viruses infect with these cells with very similar efficiencies. To correct for variations in titers between virus preparations, we chose to use this cell line as the standard, and the ratios of the titers of the K2M/K2 viruses were all normalized to the ratio of titers in this cell line. In PCC4 and PCC4 SCRAM cells, the titer of the K2 virus was strongly repressed (9- and 7.8-fold, respectively) over that of the K2M virus (Fig. 4B), demonstrating that these cells possess RBS silencing activity. As has been reported, the level of repression mediated by the PBS^{Lys-1,2} was considerably lower than that of repression mediated by the PBS^{Pro}, which has a repression in these cells of ≈ 70 -fold (13, 23). In the TRIM28 knockdown cells (PCC4 111), however, there was a near complete relief of repression, with a repression of only 1.7-fold (see Fig. 4B). The datasets for each of several individual experiments are presented in [supporting information \(SI\) Table S1](#). These results confirm that knockdown of TRIM28 causes a significant decrease in RBS-dependent silencing of PBS^{Lys-1,2}, and thus strongly suggest that TRIM28 is required for this process.

Discussion

We have demonstrated that MLV PBS^{Pro}-mediated restriction in embryonic cells requires TRIM28 (13, 19). In this article we have extended these observations to show that TRIM28 is also required for the specific restriction of PBS^{Lys-1,2}-containing viruses in embryonic cells (see Fig. 4B). We observe that the TRIM28-containing complex that recognizes the PBS^{Lys-1,2} sequence is of a similar size (as judged by EMSA migration) as the PBS^{Pro} binding complex (see Fig. 1A), and can be similarly supershifted with an anti-TRIM28 antibody (see Fig. 1A). The DNA recognition components of the complex, however, appear to be different (see Fig. 2), suggesting that TRIM28 is interacting with at least two separate DNA-binding factors, each of which recognizes a different PBS sequence. Because both of these DNA-binding components presumably interact with TRIM28 and subsequently lead to transcriptional repression, KRAB-box zinc-finger proteins make attractive candidates for this activity. KRAB-box zinc-finger proteins are a large class of zinc-finger proteins (290 in the human genome) that remain largely uncharacterized. The KRAB box has previously been shown to specifically interact with TRIM28, and this interaction has been shown to induce transcriptional repression of relevant target genes (14, 15, 28). In the context of retrovirus and retroelement silencing, we expect that further biochemical analysis of the TRIM28 complex should allow identification of these different DNA-binding factors.

These data as a whole lead us to suggest a generalized model for PBS-mediated restriction of retroviruses, in which viruses with either PBS^{Pro} and PBS^{Lys-1,2}, or indeed other as yet uncharacterized PBS sequences, are silenced in embryonic cells by the specific recruitment of TRIM28 (Fig. 5). We speculate that each PBS sequence will be recognized by a different DNA-binding protein, and that these proteins will all have the ability to recruit TRIM28. TRIM28 recruitment to the newly integrated provirus will lead to silencing of LTR-driven transcription through the subsequent recruitment of HP1 and possibly also silencing machinery, such as the NuRD histone deacetylase complex and the histone H3 K9 methyltransferase ESET (see Fig. 5), although the involvement of these last two components remains to be formally established. As

Fig. 5. Model of TRIM28-mediated restriction. *Left* shows TRIM28 recruitment to several different retroviral proviruses using different PBS sequences (either PBS^{Pro}, PBS^{Lys-1,2}, or another hypothetical PBS) by differing DNA-binding proteins. *Right* shows subsequent TRIM28-mediated transcriptional silencing of the integrated provirus by the recruitment of HP1 and also possibly the recruitment of other TRIM28 interaction partners, namely ESET and the NuRD complex.

TRIM28 is ubiquitously expressed, it also seems plausible that other components of the system, likely the DNA-binding factors themselves, will be under tight developmental control to maintain this restriction only in embryonic and stem cells.

The biological purpose of PBS-mediated silencing of retroviruses is still as yet not fully elucidated. The stem cell specificity of this process suggests that it may have evolved to prevent proviral insertions into the germ line of organisms, or possibly prevent the activation of retrotransposons during germ cell development. As TRIM28 is essential for early mouse development (29), genetic analysis of this question has so far proved difficult. Identification of the PBS-DNA-binding factors, however, which presumably will be required for this process, should lead to a better understanding of this process.

The discovery that multiple PBS sequences are specifically silenced in embryonic cells through a common mechanism suggests that this mode of restriction has evolved to specifically inhibit multiple retrovirus types. To date, most studies of PBS-mediated restriction have been performed in mouse cells. However, it has been reported that PBS^{Pro}-mediated restriction also occurs in human cells (24). Thus it is likely that other mammals will also induce embryonic silencing of other PBS-containing retroviruses. Identification of the PBS-DNA binding factors from multiple species should give insight into whether different mammalian species have evolved the ability to restrict sequences other than PBS^{Pro} and PBS^{Lys-1,2}, based presumably as a result of selective pressure to exposure to such viruses at some time during their evolution. The inherited legacy of such repressive systems provide a historical record of the viral epidemics occurring during the course of mammalian evolution, and an

indication of the importance of the restriction of virus replication as a selective force.

Experimental Procedures

Cell Culture and Stable RNAi-Expressing Cell Line Production and Transduction. PCC4, Rat2, and 293T cells were cultured in DMEM with 10% FBS at 37°C in 5% CO₂. RNAi knockdown lines were as previously described (13). Viral transduction assays and virus production were also performed as described (13). Briefly, retroviral preparations were serially diluted (for titer determination), and added to PCC4 and Rat2 cells (seeded at [PCC4] 3.5×10^3 and [Rat2] 2×10^3 cells per cm² the day before transduction) in the presence of 8 μ g of Polybrene/ml. G418-containing selective media was added 48 h after transduction at 1 mg/ml for Rat2 and at 0.5 mg/ml PCC4 cells (for PCC4 cells, media were changed to 1 mg/ml after 10 days), and colonies were counted after 14–18 days of selection. Each experiment was performed in triplicate.

Preparation of Nuclear Extracts. Nuclear extracts were prepared from PCC4 cells, as described (13).

EMSAs. EMSAs were performed as before (13) with the following probes:

PBS^{Pro}: GGGGGCTCGT CCGGGATCGGGAGACCCC
 PBS^{Lys-1,2}: GGCGCCCAACGTGGGGCCGGGAGAC CCC
 PBS^{Lys-1,2(m)}: GGCGCctAACGTGGGGCCGGGAGACCCC. Sense strands only
 are shown.

Adenoviral Production/Infections. Adenoviral production and infections were performed as described (30) with modifications (31).

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