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Phosphorylation Regulates RNA Binding by the Human T-Cell Leukemia Virus Rex Protein

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The Rex protein of human T-cell leukemia virus types I (HTLV-I) and II (HTLV-II) regulates the expression of the viral structural genes and is critical for viral replication. Rex acts by specifically binding to RNAs containing sequences of the R region of the 5′ long terminal repeat. Two forms of Rex detected in HTLV-II-infected cells, p26^{rex} and p24^{rex}, differ in the extent of serine phosphorylation. Two-dimensional phosphopeptide analysis indicates that p26^{rex} is extensively phosphorylated at multiple sites. Using a sensitive immunobinding assay, we show that the phosphorylation state of Rex determines the efficiency of binding of Rex to HTLV-II target RNAs. Thus, the phosphorylation state of Rex in the infected cell may be a switch that determines whether virus exists in a latent or productive state. These studies also suggest that phosphorylation of RNA-binding regulatory proteins is a more general mechanism of gene regulation.

Human T-cell leukemia virus types I (HTLV-I) and II (HTLV-II) have been causally associated with leukemia and neurological disorders in humans (12). The HTLVs contain a unique region located 3′ to the *env* gene that encodes the *trans*-regulatory gene products, Tax and Rex, both of which are essential for viral replication (16, 50). Tax localizes to the nucleus of infected cells (22, 34, 55) and acts to increase the rate of transcription from the viral long terminal repeat (LTR) (14, 15, 21, 52), as well as from cellular genes, including interleukin 2 (42), interleukin 2 receptor α (4, 18, 33), c-*fos* (20), and granulocyte-macrophage colony-stimulating factor (45, 58). Rex induces the cytoplasmic expression of incompletely spliced viral mRNA species that encode the Gag, Pol, and Env proteins (28, 34, 48, 51); however, the exact mechanism of Rex function is still unclear. In HTLV-II, Rex regulation requires a *cis*-acting element in the R/U5 regions of the 5′ LTR (6, 48). This region, termed the Rex response element (RxRE), is predicted to form a highly ordered and stable RNA secondary structure that is both necessary and sufficient to mediate Rex regulation of viral gene expression. An analogous *cis*-acting regulatory element required for Rex regulation has also been described in the HTLV-I 3′ LTR (26, 51).

Two *rex* gene products, p26^{rex} and p24^{rex}, are produced in HTLV-II-infected cells (50, 53). Recent structural analysis of HTLV-II p26^{rex} and p24^{rex} indicated that the two proteins share the same amino acid backbone and that posttranslational modification of p24^{rex} by serine phosphorylation is responsible for conversion to p26^{rex} (25). HTLV-I encodes two *rex* gene products of 27 and 21 kDa (35, 43). The larger Rex phosphoprotein is localized primarily in the nucleus and nucleolus (35). A highly basic amino acid sequence at the amino terminus of HTLV-I Rex is responsible for subcellular targeting to the nucleus and nucleolus and is critical for Rex function (46, 54).

HTLV-II Rex protein purified from a baculovirus expression system directly binds RNAs containing the HTLV-II R region of the 5′ LTR in vitro (7, 59). HTLV-I purified Rex was also shown to directly bind to the HTLV-I RxRE (5, 8, 23). Further analyses have indicated a direct correlation between Rex-binding RxRE sequences and Rex function in vivo (5, 7). To determine the role of phosphorylation in HTLV gene regulation, we tested whether phosphorylation of HTLV-II Rex is required for efficient binding to target RNAs in vitro.

MATERIALS AND METHODS

Cells and transfection. COS cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. Cells (5×10^6) were transfected by electroporation with 25 μg of the *tax/rex* expression plasmid BC20.2 as described previously (13, 25).

Metabolic labeling and immunoprecipitation. COS cells were incubated 48 h posttransfection for 30 min in phosphate-free RPMI 1640 medium supplemented with 10% fetal calf serum. $^{32}P_i$ (specific activity, 285 Ci/mg of P, 10.5 TBq/mg of P) (ICN Biochemicals, Inc.) was added (1 mCi/ml), and the cultures were incubated for 3 h. Cells were lysed in RIPA buffer (0.05 M Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate [SDS], 1.0% Triton X-100, 0.15 M NaCl, 2.0 mM phenylmethylsulfonyl fluoride). The lysates were then clarified by centrifugation at 100,000 × *g* for 45 min at 4°C.

Rex was immunoprecipitated from cell lysates with an antibody directed against the COOH-terminal tridecapeptide sequence encoded by *rex* (53). Immune complexes were collected with protein A-Sepharose (Pharmacia), solubilized in Laemmli sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

Western blot (immunoblot) analysis. Proteins were resolved by SDS-PAGE (10% polyacrylamide) and then electrophoretically transfered to nitrocellulose membrane (Schleicher & Schuell). The transfer was performed in 25 mM Tris–190 mM glycine–20% methanol for 1 h at 60 V with a Bio-Rad transblot apparatus. Radiolabeled proteins were visualized by direct autoradiography. Membranes containing nonradiolabeled proteins were incubated for 1.5 h at room

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temperature in 1% (wt/vol) bovine serum albumin (BSA)–0.01 M Tris (pH 7.2)–0.15 M NaCl–0.01% Tween 20. Rex-specific antiserum (1:100 dilution) was then added and incubated overnight at room temperature. After washing, the membrane was incubated with anti-rabbit immunoglobulin coupled to alkaline phosphatase (ICN Biochemicals) and developed with bromo-chloro-indolyl phosphate and nitro-blue tetrazolium.

Two-dimensional phosphopeptide map analysis. $p26^{rex}$ phosphorylated in vivo was fractionated on SDS–10% polyacrylamide gels, transferred to nitrocellulose, and detected by autoradiography. Sample preparation and two-dimensional peptide mapping were performed as previously described (11, 32, 37). Briefly, pieces of membrane containing radiolabeled Rex were incubated in 0.5% polyvinylpyrrolidone in 100 mM acetic acid for 30 min at 37°C. The membrane was then washed three times with H_2O and twice with 0.05 M NH_4HCO_3. The membrane was then incubated with 10 μg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (Worthington) for 2 h in 200 μl of 0.05 M NH_4HCO_3 at 37°C. The supernatant containing the digested peptides was washed by repeated lyophilization and then oxidized in performic acid at 0°C. The digests were applied to thin-layer cellulose plates for two-dimensional peptide mapping by electrophoresis at pH 1.9 for 30 min at 400 V in the first dimension and then by chromatography in *n*-butanol–pyridine–acetic acid–H_2O (75:50:15:60) in the second dimension. Radiolabeled peptides were visualized by direct autoradiography.

Rex dephosphorylation and immunobinding assays. Purified Rex from recombinant baculovirus-infected insect cells (25 nM) was incubated with bacterial alkaline phosphatase (BAP) (Bethesda Research Laboratories) at 60°C or with calf intestinal alkaline phosphatase (CIAP) (Stratagene) at 37°C for 15 min in 10 μl of 100 mM NaCl–10 mM $MgCl_2$–10 mM Tris (pH 8.0). Samples with phosphatase inhibitors contained 500 μM sodium vanadate and 200 μM NaF (Sigma). Samples were subjected to Western blot analysis to determine the effect of phosphatase treatment.

Immunobinding reactions were performed as previously described (59). Briefly, [α-^{32}P]CTP (20 mCi/ml, 800 Ci/mmol; Amersham)-radiolabeled RxRE containing sense or antisense RNA transcripts was synthesized in vitro from a pGEM-1 construct containing the RxRE (nucleotides 361 to 786 of the HTLV-II provirus), using T7 and SP6 RNA polymerase. The binding reaction consisted of approximately 50 pg of uniformly labeled RNA (approximately 15,000 cpm) and either 0.75 or 2 nM Rex in 50 μl of buffer (40 mM Tris [pH 8.0], 2 mM $MgCl_2$, 200 mM KCl, 16 μg of tRNA per ml, 20 μg of BSA per ml, 0.05% Triton X-100, 40 U of RNasin per ml). Reaction mixtures were incubated at 4°C for 15 min, and then 0.5 μl of Rex-specific antiserum was added and incubated further for 20 min. Rex-RNA complexes were collected with protein A-Sepharose and washed, and the fraction of RNA bound was determined by liquid scintillation counting.

RESULTS

Rex phosphorylation in vivo. We previously reported that HTLV-II $p26^{rex}$ is heavily phosphorylated in vivo, whereas $p24^{rex}$ phosphorylation is negligible (25). We also showed that Rex immunoprecipitated from lymphoid cells and treated with BAP resulted in loss of $p26^{rex}$ and a concomitant increase in $p24^{rex}$ (25). Previous studies have indicated that $p26^{rex}$ is phosphorylated primarily on serine residues, with some detectable phosphothreonine (25). Rex consists of 25 serines and 12 threonines. The extent of Rex phosphorylation in vivo was investigated by two-dimensional phosphopeptide map analysis after complete digestion of $p26^{rex}$ with trypsin. Eight phosphopeptides were consistently detected; two peptides contained major ^{32}P incorporation, whereas the other six were of minor intensity (Fig. 1). As predicted by the amino acid sequence of Rex, a complete tryptic digest will give rise to six peptides which contain serine residues. Thus, several of the eight peptides detected are most likely the result of incomplete digestion at trypsin-resistant sites. These results indicate that Rex is phosphorylated on multiple residues.

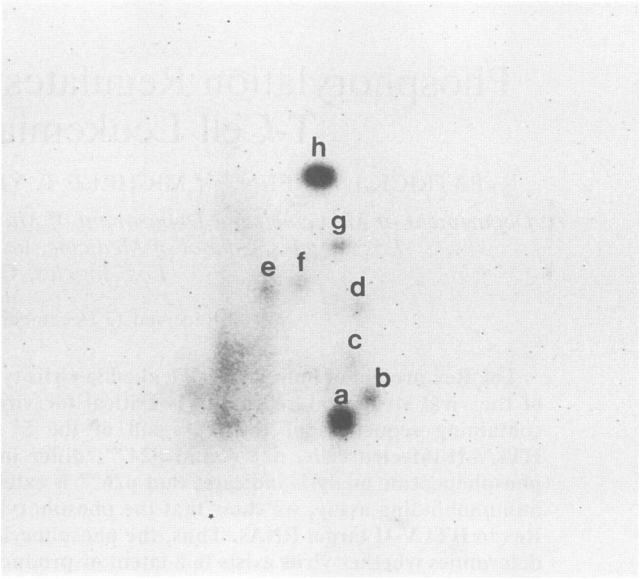

FIG. 1. Two-dimensional phosphopeptide map analysis of Rex. COS cells transfected with the *rex* expression plasmid, BC20.2, were metabolically labeled with $^{32}P_i$, and cell lysates were prepared as described previously (25). Lysates were immunoprecipitated, subjected to SDS-PAGE, and transferred to nitrocellulose membrane as described in Materials and Methods. Pieces of membrane containing ^{32}P-labeled $p26^{rex}$ were incubated with 10 μg of TCPK-trypsin for 3 h at 37°C. Digested peptides released into the supernatant were lyophilized and resuspended in pH 1.9 buffer, spotted on cellulose plates, and separated into two dimensions as described in the Materials and Methods. Electrophoresis was from left (anode) to right (cathode), and chromatography was from bottom to top.

Baculovirus-purified Rex is phosphorylated and binds RxRE RNA in vitro. We previously copurified HTLV-II $p26^{rex}$ and $p24^{rex}$ from recombinant baculovirus-infected insect cells (Sf9). Rex proteins expressed in insect cells were identical in size to Rex ($p26^{rex}$/$p24^{rex}$) produced in human lymphoid cells and COS cells (50, 59). Baculovirus-purified Rex was treated with increasing concentrations of BAP and subjected to Western blot analysis to determine the effect of increased BAP concentrations on the relative amounts of $p26^{rex}$ and $p24^{rex}$. With increasing concentrations of BAP, there was a gradual decrease in $p26^{rex}$ and a concomitant increase in the smaller *rex* gene product (Fig. 2A). Thus, the Rex proteins produced in insect cells also represent forms of Rex that differ by phosphorylation. We recently reported that this baculovirus-purified Rex specifically binds RNAs containing the RxRE in both immunobinding and gel mobility shift assays and that Rex binding to RxRE RNA correlates with Rex function (7, 59).

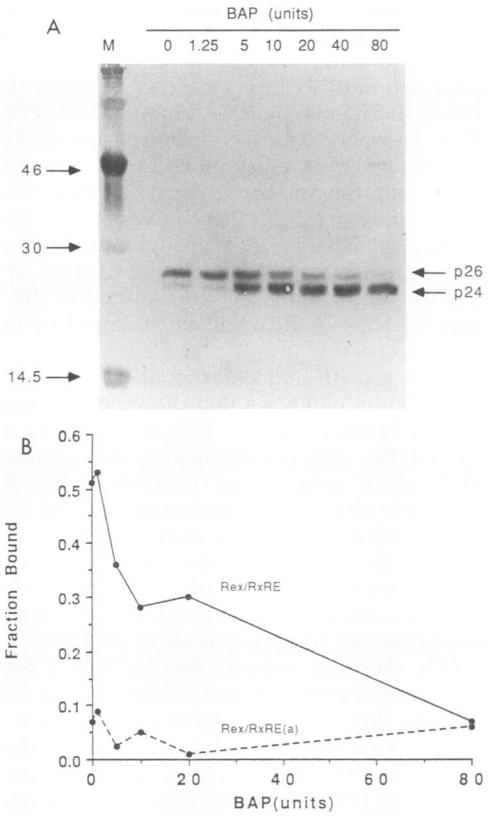

FIG. 2. Binding of HTLV-II Rex treated with BAP to RNA containing the RxRE. (A) Purified Rex from recombinant baculovirus-infected insect cells (25 nM) was incubated with BAP (0 to 80 U) at 60°C for 15 min, and samples were assayed by Western blot analysis as described in Materials and Methods. Each lane contains a total of approximately 25 ng of purified Rex. The positions of $p26^{rex}$ and $p24^{rex}$ are labeled on the right, and protein markers (in kilodaltons) (lane M) are indicated on the left. (B) Labeled sense and antisense RNA corresponding to nucleotides 361 to 786 (containing the defined RxRE) in the HTLV-II 5′ LTR was synthesized in vitro as described in Materials and Methods. Rex (25 nM) was preincubated with BAP (0 to 80 U) and tested for its ability to bind these RNAs. The immunobinding reaction consisted of approximately 50 pg of uniformly labeled RNA (approximately 15,000 cpm) and 2 nM Rex and was performed as described in Materials and Methods. —●—, Rex-RxRE sense RNA; --●--, Rex-RxRE(a) antisense RNA.

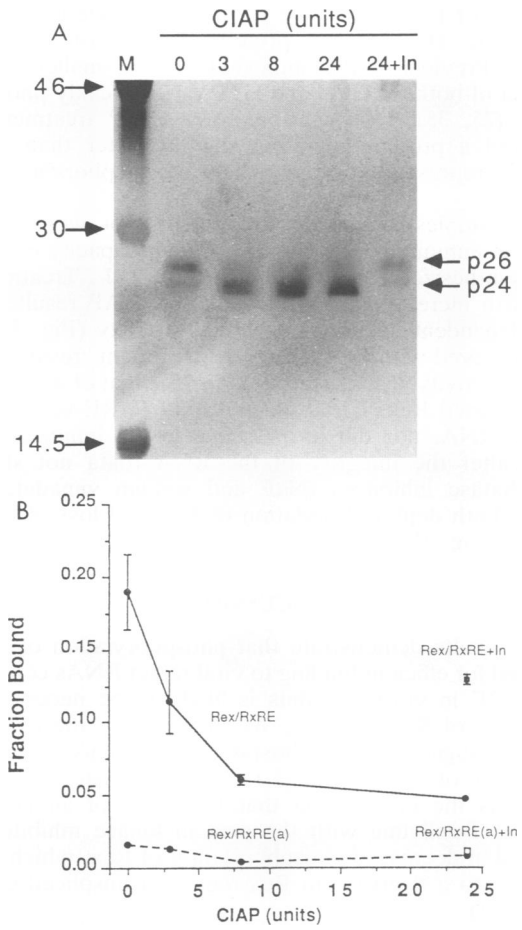

FIG. 3. Binding of HTLV-II Rex treated with CIAP to RNA containing the RxRE. (A) Purified Rex (25 nM) was incubated with CIAP (0 to 24 U) at 37°C for 15 min as described in Materials and Methods. Samples with phosphatase inhibitors (In) contained 500 μM sodium vanadate and 200 μM NaF (Sigma). Samples of Rex protein after CIAP treatment (0 to 24 U) were assayed by Western blot analysis as described in Materials and Methods. Each lane contains a total of approximately 5 ng of purified Rex. The positions of $p26^{rex}$ and $p24^{rex}$ are labeled on the right, and protein markers (in kilodaltons) (lane M) are indicated on the left. (B) Rex protein samples treated and analyzed above were tested in duplicate for their ability to bind RxRE-containing RNA. The immunobinding reaction consisted of approximately 50 pg of uniformly labeled RNA (approximately 15,000 cpm) and 0.75 nM Rex and was performed as described in Materials and Methods. —●—, fraction bound of the RxRE sense RNA; --●--, fraction bound of the RxRE(a) antisense RNA; ■ and □, fraction bound of the RxRE sense RNA and RxRE(a) antisense RNA, respectively, after treatment of Rex with CIAP in the presence of phosphatase inhibitors (500 μM sodium vanadate and 200 μM NaF).

Rex dephosphorylation decreases the efficiency of binding to RxRE RNA in vitro. Baculovirus-purified Rex treated with BAP was tested by using a very sensitive immunobinding assay to determine whether phosphorylation is important for Rex binding to target RNAs. Treatment of Rex with increasing concentrations of BAP resulted in a dose-dependent decrease in binding activity. At the highest BAP concentration tested (80 U), binding activity was 15 to 25% of wild-type Rex-binding activity (Fig. 2B). Phosphatase treatment had no effect on the low nonspecific binding of Rex to antisense RxRE RNA. BAP treatment of the analogous RNA-binding protein of human immunodeficiency virus type 1 (HIV-1), Rev (27), had no effect on its ability to bind RNAs containing the Rev response element in similar assays (data not shown). Thus, loss of phosphate groups from Rex decreases the specific binding activity of Rex for target RNAs containing the RxRE. However, it is noteworthy that the dephosphorylated form of Rex still has residual binding activity of 15 to 25% that of wild-type phosphorylated Rex.

We also investigated the effect of treatment of purified Rex with CIAP. CIAP treatment can be performed at physiological temperatures, and CIAP can be efficiently inactivated by inhibitors. Treatment of purified Rex with CIAP resulted in loss of $p26^{rex}$, with a concomitant increase in $p24^{rex}$ (Fig. 3A). CIAP treatment of purified Rex in the presence of protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, and leupeptin) also resulted in loss of $p26^{rex}$, indicating

that loss of $p26^{rex}$ was due to dephosphorylation and not proteolytic cleavage or protein degradation (data not shown). Previous studies indicated that the smaller *rex* gene product of both HTLV-I and HTLV-II is weakly phosphorylated (25, 35). On occasion, after CIAP treatment, we observed a protein migrating slightly faster than $p24^{rex}$, possibly representing a completely dephosphorylated form of Rex.

Rex samples analyzed by Western blot were directly tested in immunobinding assays for their capacity to bind in vitro-synthesized RNA containing the RxRE. Treatment of Rex with increasing concentrations of CIAP resulted in a dose-dependent decrease in binding activity (Fig. 3B). As was observed with Rex after BAP treatment, residual Rex-binding activity is consistently 15 to 25% that of wild-type or mock-treated Rex. CIAP did not bind RxRE sense or antisense RNA, nor did its presence in the immunobinding assay alter the integrity of the RNA (data not shown). Phosphatase inhibitors (NaF and sodium vanadate) prevented both dephosphorylation of Rex and loss of binding activity (Fig. 3B).

DISCUSSION

Our results demonstrate that phosphorylation of Rex is required for efficient binding to viral target RNAs containing the RxRE in vitro and thus is likely to be necessary for regulation of Rex function. We provide, for the first time, evidence suggesting that phosphorylation plays a role in the regulation of RNA-binding factors. Consistent with our finding is the observation that treatment of an HTLV-I-infected T-cell line with the protein kinase inhibitor H-7 resulted in decreased phosphorylation of Rex, which corresponded to a decrease in Rex-regulated unspliced *gag/pol* mRNA (1).

Protein phosphorylation has also been shown to regulate the activity of some DNA-binding factors. Phosphorylation of c-Jun, which controls transcriptional activation of the AP-1-responsive genes, negatively regulates its DNA-binding activity (10). The DNA-binding activity of c-Myb for its response element is also inhibited by phosphorylation (38). The DNA-binding activities of the E2F and E4F transcription factors induced by adenovirus infection are increased by phosphorylation (3, 49), and the DNA-binding activity of the serum response factor is induced by phosphorylation (41). Thus, protein phosphorylation can be a positive or negative regulator of gene expression by directly controlling nucleic acid-binding activity.

Phosphorylation of Rex may affect RNA binding by several possible mechanisms. Phosphorylation alters protein charge, which may result in a conformational change unmasking the RNA-binding domain. Both Rex and HIV-1 Rev contain an arginine-rich motif that has been identified in several RNA-binding proteins (36). This region is also required for nucleolar localization and is interchangeable between Rev and Rex (29). A recent study investigating the binding of Rev/Rex chimeras to Rev response element-containing RNA indicates that amino acid residues outside the nuclear localization signal functionally contribute to the binding of Rex to the Rev response element, suggesting different domain organizations of Rev and Rex with respect to subcellular localization and RNA recognition (9). Phosphorylation of the carboxy terminus of c-Fos is required for transcriptional repression of its own promoter, and this requirement for phosphorylation can be offset by the introduction of a net negative charge in the carboxy terminus (47). Another possibility is that Rex, like HIV-1 Rev, acts as an oligomer (19), and phosphorylation may be necessary for the oligomerized state.

Phosphorylation of Rex may have consequences in addition to positively regulating RNA binding. For HTLV-I, the larger Rex phosphoprotein is localized primarily in the nucleus, although also present in lesser amounts in the cytoplasm, while the smaller *rex* gene product is detected only in the cytoplasm (35). If these proteins are analogous to HTLV-II Rex, phosphorylation of Rex may be required for the proper compartmentalization in the nucleus or may be critical for its stabilization once transported to the nucleus. Mutational analysis of Rex will be required to test these possibilities.

Normal cell growth and differentiation are regulated by signal transduction pathways that often involve the antagonistic effects of cellular kinases and phosphatases. For both HTLV and HIV, efficient replication is dependent on a balanced level of spliced and unspliced viral RNA for synthesis of new virion components and genomic RNA. Rex and HIV Rev binding to RNAs containing their respective response elements is critical for this regulation; however, the precise mechanism of this regulation is not clear (2, 39, 40). HIV-1 Rev is a nuclear phosphoprotein, although for Rev, phosphorylation has no apparent effect on RNA binding or function (17). Our data indicate that for HTLV Rex, phosphorylation is critical for efficient binding. These differences may reflect the differing lifecycles of these viruses; HIV-1 is dependent on a productive and possibly lytic infection for spread, whereas HTLV is a tightly cell-associated transforming virus. In HTLV-infected cells, both phosphorylated and nonphosphorylated forms of Rex are present. The concentration of phosphorylated Rex would be dependent on the balance of cellular kinase and phosphatase activities, which are highly regulated both positively and negatively by environmental stimuli (30, 31). Our results would predict that in the absence of phosphorylated Rex, less incompletely spliced mRNA (*env* and *gag/pol* mRNAs) would be expressed, and that *tax/rex* mRNA would predominate. In such a state, it is conceivable that production of the *trans*-regulatory protein Tax, thought to be important in viral transformation (24, 44, 56, 57), could maintain the proliferative state of the cell and thus replicate the proviral form of the virus through cell division. Since no viral structural proteins would be produced, the immune response would be more restricted. Phosphorylation of Rex would result in a shift to the productive phase of HTLV replication, with an increase in the synthesis of viral structural proteins, virion production, and recruitment of newly infected cell clones. Therefore, phosphorylation and dephosphorylation of Rex could result in highly regulated HTLV replication.

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