Phosphorylation of ORF1p is required for L1 retrotransposition

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Although members of the L1 (LINE-1) clade of non-LTR retrotransposons can be deleterious, the L1 clade has remained active in most mammals for ∼100 million years and generated almost 40% of the human genome. The details of L1–host interaction are largely unknown, however. Here we report that L1 activity requires phosphorylation of the protein encoded by the L1 ORF1 (ORF1p). Critical phospho-acceptor residues (two serines and two threonines) reside in four conserved proline-directed protein kinase (PDPK) target sites. The PDPK family includes mitogen-activated protein kinases and cyclin-dependent kinases. Mutation of any PDPK phospho-acceptor inhibits L1 retrotransposition. The phosphomimetic aspartic acid can restore activity at the two serine sites, but not at either threonine site, where it is strongly inhibitory. ORF1p also contains conserved PDPK docking sites, which promote specific interaction of PDPKs with their targets. As expected, mutations in these sites also inhibit L1 activity. PDPK mutations in ORF1p that inactivate L1 have no significant effect on the ability of ORF1p to anneal RNA in vitro, an important biochemical property of the protein. We show that phosphorylated PDPK sites in ORF1p are required for an interaction with the peptidyl prolyl isomerase 1 (Pin1), a critical component of PDPK-mediated regulation. Pin1 acts via isomerization of proline side chains at phosphorylated PDPK motifs, thereby affecting substrate conformation and activity. Our demonstration that L1 activity is dependent on and integrated with cellular phosphorylation regulatory cascades significantly increases our understanding of interactions between L1 and its host.

proline-directed protein kinase | LINE-1 | peptidyl prolyl isomerase 1 | retrotransposon | Pin1

L1 (or LINE-1) activity over the last ∼100 million years of primate evolution has generated ∼40% of the human genome (1, 2); thus, succeeding families of L1 elements are the main drivers of genetic expansion. These autonomously replicating elements convert their RNA transcripts and those of other genetic elements, particularly SINEs, into genomic DNA (3). A generic L1 element is 6–7 kb and contains the following: a 5′ UTR; ORF1, which encodes the coiled-coil mediated trimeric nucleic acid chaperone protein ORF1p; ORF2, which encodes a DNA endonuclease and reverse-transcriptase ORF2p; and a 3′ UTR terminated in a polyA sequence (reviewed in refs. 3 and 4). ORF1p, ORF2p, and L1 RNA form ribonucleoprotein particles (RNPs) that are likely intermediates in L1 retrotransposition (5–8). The L1-encoded proteins ORF1p and ORF2p are essential in cell culture-based retrotransposition assays (9) and in vitro assays using RNPs from cells transfected with L1 retrotransposition vectors (7). Although the role of ORF1p in retrotransposition is not known, mutations that affect its nucleic acid binding and chaperone activities can inactivate L1 (7, 9, 10).

L1 activity can damage DNA (11), can generate genetic diversity and rearrangements (12–16), and is activated in certain tumors (17–19) and other somatic cells (20), including neuronal cells (21–23). Despite being deleterious (24, 25), with at times catastrophic effects (26, 27), novel L1 families continue to evolve in modern mammals (15, 28–30), at least in some cases in response to evolving mammalian defensive measures (31). The existence of strong negative selection (24, 25) and robust host repressive mechanisms, which include methylation of L1 DNA (26), inhibition by APOBEC cytosine deaminases (32–35), and repression by Argonaute protein-mediated RNAi (36), support a parasitic nature of L1 elements (37); however, the overall effect of L1 on mammalian evolution and biology, and how L1 interacts with and persists in the host, remain unanswered questions.

Early reports suggested that ORF1p was phosphorylated (6, 38). Recent studies have shown that phosphorylation-related proteins can be coimmunoprecipitated with ORF1p or L1 RNPs (39, 40). In addition, the mitogen-activated protein kinase (MAPK) p38 has been implicated in L1 activation by environmental toxins (41, 42), and its expression can be increased by exogenous ORF1p (43). Although large-scale proteomic studies can only identify a subset of phospho sites (44), a phosphoproteomic study of human embryonal stem cells, in which L1 is active, identified an ORF1p fragment phosphorylated on S18 (45). This finding suggested to us that ORF1p phosphorylation might be required for L1 activity.

To investigate the role of ORF1p phosphorylation in retrotransposition, we used LC-MS/MS to determine the phosphorylation state of ORF1p purified from insect and HeLa cells and identified a total of 14 high-confidence phospho residues. Mutational analysis showed that highly conserved proline-directed protein kinase (PDPK) target sites and docking motifs are critical for L1 retrotransposition. PDPKs specifically phosphorylate serines or threonines with proline in the +1 position (S/T-P motifs) (46). Docking motifs on PDPK substrates ensure efficient kinase targeting, and phosphorylation of docking motifs by protein kinase A (PKA) can regulate PDPK binding (46, 47). Two PDPK docking motifs in ORF1p contain a predicted PKA site, and we show that mutation of either of these sites also inhibits

Significance

All organisms contain transposable DNA elements (TEs) that can be seriously deleterious. The dominant TE in mammals, the L1 (LINE-1) retrotransposon, has generated approximately 40% of the genome. L1 encodes two proteins, ORF1p and ORF2p, that are required for L1 retrotransposition. We show here that phosphorylation of ORF1p is required for this process. These results significantly advance our understanding of retrotransposition and indicate that L1 activity is integrated with, and thus potentially can perturb, host cellular signaling pathways. Thus, the effects of L1 may extend well beyond those of genome alteration as is currently thought.

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Data deposition: MS/MS ORF1p phosphorylation data are available at yped.med.yale.edu/repository under project name ORF1p.

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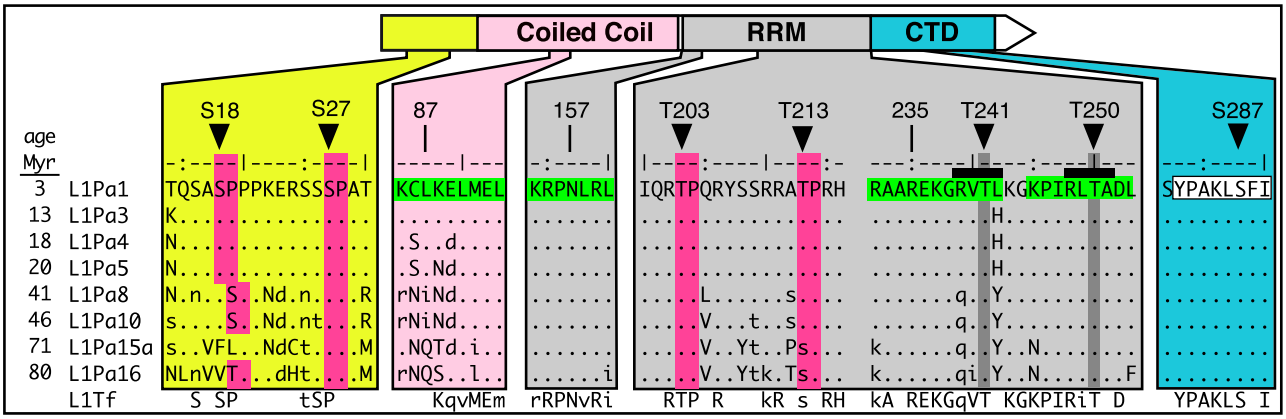

Fig. 1. PDPK motifs are conserved in ORF1p. ELM identified in the modern L1Pa1 ORF1p consensus sequence (28): four PDPK S/T-P targets (dark-pink columns); four PDPK docking motifs approximated by (R/K)•••h•h, where • is any residue and **h** is a hydrophobic residue (green); within PDPK docking motifs, two PKA motifs, R•(S/T)-not-P (black bars) with the target sites T241 and T250; and a putative PP1 docking site (white). The consensus sequences of the ancestral L1Pa families and mouse L1Tf family were reported previously (30, 73, 74). RRM, RNA recognition motif (light gray); CTD, C-terminal domain (teal) (75).

L1 retrotransposition. Finally, we show that the serine PDPK sites in ORF1p mediate an interaction with the proline isomerase Pin1, an essential component of PDPK-mediated regulatory pathways. Pin1 binds to phosphorylated S/T-P motifs and, via proline isomerization, induces significant conformational changes, which can affect activity, stability, protein–protein interactions, phosphorylation state, and susceptibility to further posttranslational modifications (48, 49). Taken together, these results demonstrate a role for PDPK(s) in L1 retrotransposition and indicate that L1 activity is integrated into host kinase pathways, with potentially far-reaching effects on cellular function.

Results

ORF1p Is Phosphorylated on Multiple Residues. Analysis of the primary amino acid sequence of ORF1p with ELM, the database of eukaryotic linear motifs (50), revealed a number of highly conserved kinase motifs, some of which are shown in Fig. 1. These include four S/T-P PDPK target sites: two serine sites, S18 and S27, lying in the N-terminal region, and two threonine sites, T203 and T213, in the RNA recognition motif (RRM) of ORF1p (51). ORF1p also contains multiple PDPK docking motifs, two of which contain predicted PKA sites at highly conserved T241 and T250. In addition, ORF1p contains a docking site for protein phosphatase 1 (PP1). Mouse ORF1p contains potential phosphorylation sites that correspond to positions S18, S27, S119, T203, T250, S254, and S287 in human ORF1p (Fig. 1 and Table 1), suggesting that these sites have been conserved for at least 120 million years, the estimated time at which rodents and primates diverged (52).

LC-MS/MS analysis of ORF1p purified from High Five insect or HeLa cells recovered peptides corresponding to ∼80% of ORF1p (SI LC-MS/MS data). Table 1 lists the high-confidence or manually confirmed phospho residues identified in ORF1p purified from each cell type. Confidence is based on two scores: the Mowse score, which ranks the confidence with which the peptide sequences match those of a given protein database (53), in our case NCBInr, and the Mascot Delta (MD) score, which ranks the confidence of phosphate assignment to a particular residue within a given peptide (54).

Three of the four PDPK target sites (S18, S27, and T203) were phosphorylated in both cell types and further verified for ORF1p-Flag by manual inspection of MS/MS spectra to confirm peptide identification and phospho site assignments (Fig. S1). Peptides containing the fourth PDPK site, T213, were either not recovered (HeLa cells) or not unambiguously identified (insect cells; *SI Results*) so the phosphorylation state of T213 remains unknown. Phosphorylation of only one of the two predicted PKA sites in the PDPK docking motifs—T250—was detected by LC-MS/MS, and only in insect cells.

In addition to the high-confidence sites listed in Table 1, LC-MS/MS identified other phosphorylation sites that could represent accurate assignments, as was the case with T203 (*SI Results*). Thus, our mutational analysis included the following actual or potential phosphorylation sites (those in bold are listed in Table 2): T14, S16, **S18**, S25, S26, **S27**, T30, S33, S50, Y52, S53, S119, S145, S166, **T203**, **T250**, S254, S281, S287, and S290, as well as **T213** and **T241**, although the latter two were not identified as potential phosphorylation sites by LC-MS/MS.

Table 1. MS/MS-detected phospho residues in ORF1p

Residue	Insect	HeLa	L1Pa*	circa My
S16	X		6	27
S18†	X	X	11	53
S27†	X	X	16	80
S50	X	X	6	27
S53	X	X	8	41
S119†	X		16	80
S145	X	X	8a	42
S166	X	X	8	41
T203†	X	X	16	80
T250†	X		16	80
S254†	X		16	80
S281	X		16	80
S287†	X		16	80
S290	X		16	80

X indicates phosphorylated L1Pa1 residues in peptides identified with ≥95% confidence, except T203, which was independently confirmed by manual inspection of the MS/MS spectra in HeLa cells. Only phospho residues with a rounded Mascot Delta Score ≥10 are shown, which corresponds to ≥91% confidence in the assignment of the phosphate to the listed residue, vs. ≤9% confidence for an alternate site in the peptide.

*The column labeled "L1Pa" indicates the particular ancestral L1Pa family in which the indicated residue can first be detected. For example, the S16 ortholog of L1Pa1 is present in L1Pa6, which was active ∼27 Mya.

†Homologs identified in mouse.

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Table 2. Effects of ORF1p mutations on retrotransposition

	Relative colony area*		Average
PDPK target			
S18A	0.21	0.23	0.22
S18D	0.74	0.71	0.73
S27A	0.40	0.36	0.38
S27D	0.83	0.88	0.85
S18A/S27A	0.01	0.02	0.02
S18D/S27D	0.40	0.41	0.41
T203G	0.01	0.01	0.01
T203S	1.06	1.01	1.03
T203D	0.00	0.00	0.00
T213G	0.29	0.26	0.27
T213S†	0.67	0.66	0.66
T213D	0.00	0.01	0.01
PDPK docking			
T241A	0.00	0.00	0.00
T250G	0.14	0.14	0.14
PDPK proline			
P19A	0.26	0.26	0.26
P28A	0.60	0.54	0.57
P204A	0.00	0.00	0.00
P214A	0.40	0.29	0.30

*Colony area is the percentage of well area covered by G418-resistant foci. All of these data, except that for T213S, were obtained at the same time along with eight determinations of WT, which yielded a mean % colony area of 48.6 ± 3.6.

†T213S was assayed at a different time, and the mean % colony area for its WT (four determinations) was 39.5 ± 3.9. The WT mean was set at 1.00 for each dataset.

PDPK Sites in ORF1p Are Required for Retrotransposition. We tested the ORF1p mutants in L1 retrotransposition in HeLa cells using a previously described assay (9) with the engineered L1 construct pRTC-puro (Fig. 2). This vector contains a neomycin reporter gene (*neo*), interrupted by an intron, in the reverse orientation to L1. The *neo* gene becomes functional only after a cDNA copy of an appropriately spliced RNA is inserted into the genome, that is, a retrotransposition event, which rendered the cells resistant to G418, an analog of neomycin. After selection with G418, cells were fixed, stained, and quantified using the ImageJ plugin ColonyArea (55). We determined transfection efficiency by puromycin selection to kill all nontransfected cells, with surviving cells fixed and stained as described above (Fig. S2).

We separately mutated the 22 actual or potential ORF1p phosphorylation sites listed above and found that mutations in all six PDPK-relevant sites (four target, two docking) seriously inhibited retrotransposition (Tables 1 and 2 and Fig. 3). In contrast, of the 16 non-PDPK sites, only S119, located in the first trimerization motif in the coiled coil (56), and S287 in the PP1 site produced >70% inhibition, despite the fact that some of these residues are highly conserved and were found to be phosphorylated in HeLa cells with high confidence, i.e., S50, S53, S145, and S166 (Table 1 and Fig. S3). The bias of inhibitory mutations toward the PDKP-relevant sites was highly significant ($P = 0.0004$, Fisher exact test).

Regarding the serine PDPK target site mutations, alanine substitution of S18 or S27 decreased retrotransposition by 60–80%, but the double mutant S18A/S27A was barely active. Phosphomimetic substitutions S18D and S27D restored L1 function to >70% and 85% of WT, respectively, and S18D/S27D also rescued S18A/S27A, but to a lesser extent (Table 2). These results suggest that phosphorylation of both S18 and S27 are required for maximal retrotransposition activity, a result consistent with the known cooperative and synergistic effects of multisite phosphorylation; i.e., functional outcomes can be scaled based on gradients of phosphorylation events (57–59).

Mutation of either of the two threonine PDPK sites located in the RRM of ORF1p also strongly inhibited L1 activity. Mutation of T203 to glycine almost abolished retrotransposition, and although the phosphorylation state of T213 was not determined, T213G inhibited retrotransposition by ∼75%. The conservative T203S and T213S mutations, which preserve a PDPK target motif, exhibited WT and 65% of WT activity, respectively; however, in contrast to the serine sites, phosphomimetic substitutions at either threonine site eliminated retrotransposition. Thus, whereas phosphorylatable residues at these sites appear to be essential, a permanently acidic moiety at either threonine inactivates the protein, raising the possibility that reversible phosphorylation at these sites may be necessary during retrotransposition.

Reversible phosphorylation at the C-terminal PDPK threonines would necessitate phosphatase activity, and, as shown in Fig. 1, ELM identified a putative PPI docking motif (P283-I289) that overlaps the highly conserved sequence 282-YPAKLS-287. Previous studies have shown that mutations of this site to 282-AAALA-287 inhibit retrotransposition and the formation of L1 RNPs (7–9). Phosphorylation is not known to regulate PPI binding, but S287 was phosphorylated in insect cells, and S287A decreased retrotransposition by ∼80% (Fig. S3). Whether S287 phosphorylation affects PPI binding remains to be clarified.

Proline residues at PDPK S/T-P target sites are essential for substrate recognition and kinase activity. Therefore, if phosphorylation of these motifs is required for L1 activity, then mutating the proline, a critical component of the motif, should also inhibit retrotransposition. Fig. 3 and Table 2 show that P19A, P204A, and P214A parallel the inhibitory effects of S18A, T203G, and T213G, respectively; however, P28A was somewhat less inhibitory than S27A. Of note, in the absence of P28, the arginine at −3 relative to S27 creates a canonical motif for an AGC kinase, which does not tolerate proline in the +1 position (60–62). Thus, it is possible that S27 was phosphorylated by a non-PDPK kinase when P28 was mutated.

Mutations of PDPK Docking Motifs Inhibit L1 Activity. As described earlier, ELM identified multiple PDPK docking motifs in ORF1p, two of which are potentially regulated by putative PKA sites T241 and T250 (Fig. 1). PDPK docking sites are thought to reside in close spatial proximity to target motifs, and the docking sites containing T241 and T250 lie very close to T203 and T213 in the ORF1p crystal structure (56). Although neither T241 nor T250 was found to be phosphorylated in HeLa cells, T250 was phosphorylated in insect cells. Because LC-MS/MS can miss

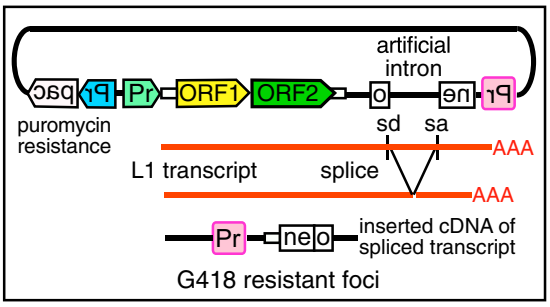

Fig. 2. The retrotransposition vector contains a full-length modern L1.3 element, an active member of the L1Pa1 family (not to scale) (76) driven by the SV40 early promoter (Pr, light green); a neomycin-resistance gene (*neo*) in reverse orientation interrupted by a sense artificial intron with splice donor (sd) and acceptor (sa) sites and driven by the Rous sarcoma virus LTR promoter (Pr, pink); and a puromycin-resistance gene (*pac*) driven by the CMV promoter (Pr, blue).

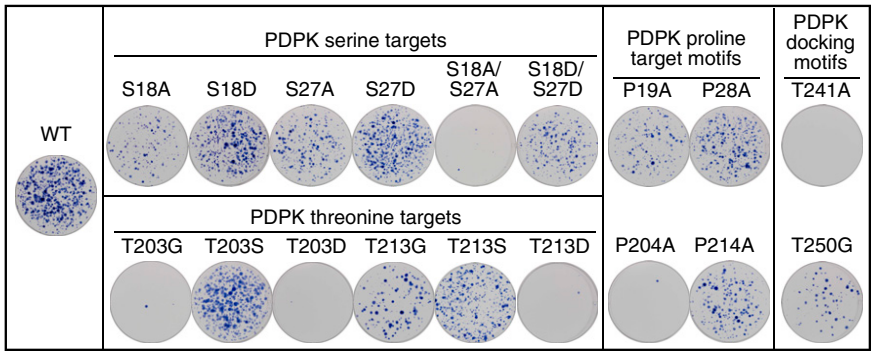

Fig. 3. Mutation of PDPK target and docking sites in ORF1p inhibit retrotransposition. Wells show Giemsa-stained foci generated from HeLa cells transfected with L1 retrotransposition vectors that express WT or the indicated mutant ORF1p. These assays were carried out in duplicate; Table 2 presents the quantified data.

low-abundance and reversible phospho residues (63), we determined the effect of mutations at each site. Substitution of T241 with alanine eliminated L1 activity, and glycine substitution at T250 reduced L1 activity by ~85% (Fig. 3 and Table 2). Whether the decrease in L1 retrotransposition was related to altered phosphorylation or structural perturbation of the PDPK docking sites, or to some other cause, is not known.

ORF1p Mutants Are Expressed in HeLa Cells. PDPK target site mutations did not prevent the expression of ORF1p in HeLa cells. ORF1p mutants that were inactive in retrotransposition could be expressed and purified for further analysis in vitro (Fig. 4 and Fig. S4). In addition, the proline and docking site mutant proteins were readily detected by Western blot analysis (Fig. S5).

RNA Annealing Activity of ORF1p Is Unaffected by PDPK Mutations. RNA binding and nucleic acid chaperone activity are important biochemical properties of ORF1p, and facilitated annealing of RNA is an essential component of chaperone activity (64); therefore, we compared this activity for WT and mutant ORF1p using FRET (65). In this assay, RNA annealing allows transfer of emission energy from an excited donor fluorophore on one RNA strand to an acceptor fluorophore on its complementary strand (Fig. 4*A*). As annealing progresses, acceptor emission increases, with a concomitant decrease in donor emission. The FRET ratio (acceptor emission/donor emission) is plotted against time and fit to the exponential equation shown in Fig. 4*C* to obtain the annealing rate constant, k_{ann}. The k_{ann} of WT and S18D/S27D ORF1p, both active in retrotransposition, were not statistically different from those of the retrotransposition incompetent mutants S18A/S27A, T203G, T203D, and T213D (Fig. 4*C*). Thus, the ability of ORF1p to anneal RNA in vitro is not dependent on phosphorylation of its PDPK motifs.

ORF1p PDPK Target Sites Mediate an Interaction with Pin1. Pin1 is an essential component of numerous PDPK-mediated regulatory pathways (48, 49). This highly conserved protein specifically binds phosphorylated PDPK sites and catalyzes the *cis*/*trans* conversion of the proline side chains of S/T-P motifs, leading to conformational changes of its substrates with significant and varied functional outcomes (48, 49). To determine whether Pin1 could target ORF1p, we performed GST pull-down assays using lysates obtained from HeLa cells transfected with expression plasmids for WT ORF1p-Flag and the indicated mutants (Fig. 5). WT ORF1p bound to GST-Pin1, but not GST, and mutation of either the S18 or S27 PDPK sites (phosphorylated in WT ORF1p) strongly impaired Pin1 binding. The double mutant S18/27A almost completely eliminated the interaction with Pin1. These results indicate that in whole-cell lysates, the majority of Pin1 binding is mediated by S18 and S27. The phosphomimetic S18D/S27D, which restored approximately 40% of retrotransposition activity, barely if at all increased Pin1 binding. This finding is not surprising, given the central role of the phosphate group in Pin1 binding (66), and is consistent with failure of a phosphomimetic to restore Pin1 binding to mutated serine PDPK target sites on the transcription factor Nanog (67).

Discussion

Given the dominant role of L1 retrotransposons in the structure and composition of most mammalian genomes, our lack of knowledge of the interface between L1 elements and their hosts represents a major void in our knowledge of mammalian biology. Therefore, our findings establishing that phosphorylation of

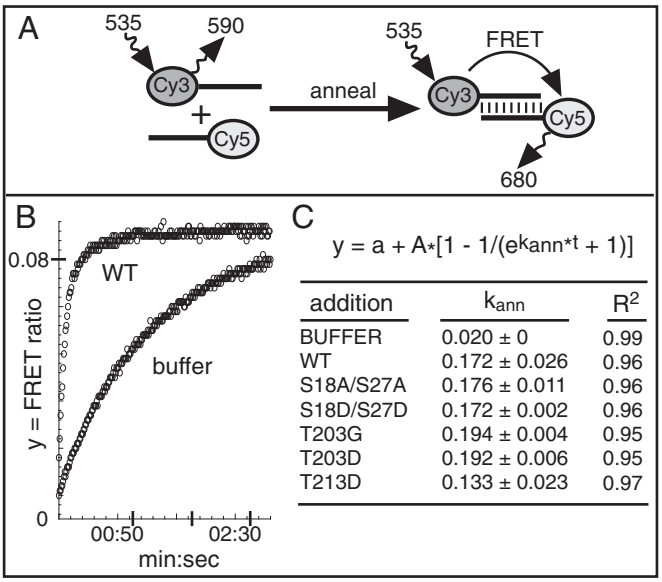

Fig. 4. ORF1p PDPK mutants can anneal RNA. (*A*) Schematic of the FRET reaction showing complementary oligonucleotides labeled with the donor (Cy3) and the acceptor (Cy5) fluorophores and their respective excitation and emission wavelengths, as described in the text. (*B*) Curves for the annealing reaction with buffer or purified WT ORF1p. The FRET ratio (*y* axis) changes as a function of time (*x* axis). All reactions were carried out in triplicate. (*C*) Mean ± SD rate constants, k_{ann}, were derived from a least squares fit of the FRET ratio to the indicated rate equation. Pairwise *t* tests showed that the k_{ann} values of WT and ORF1p mutants were not statistically different; *P* values: S18A/S27A, 0.825; S18D/S27D, 0.984; T023G, 0.274; T203D, 0.301; T213D, 0.124.

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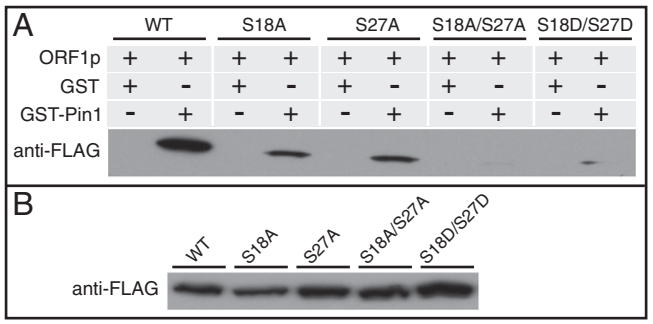

Fig. 5. PDPK sites in ORF1p mediate binding to Pin1. (*A*) GST or GST-Pin1 were incubated with whole-cell lysates from HeLa cells transfected with expression plasmids for ORF1p WT or indicated mutants. (*B*) Relative amount of ORF1p-Flag per 125 μg of whole-cell lysate for each ORF1p construct.

ORF1p by PDPKs is essential for L1 retrotransposition constitute a major advance in our understanding of L1–host interactions.

Mutation of any of the six PDPK sites seriously inhibited or eliminated retrotransposition, whereas mutations of only two of the 16 non-PDPK sites were inhibitory. This strong bias (P = 0.0004, Fisher's exact test) supports our conclusion that phosphorylation of ORF1p by PDPKs is necessary for L1 activity. In addition, mutating the prolines of each S/T-P motif, a critical element required for effective PDPK recognition, generally recapitulated the inhibitory effects of the phospho site mutations. Moreover, the rescue of activity by phosphomimetic substitutions at the N-terminal serines indicates that the relevant biochemical property at these PDPK sites is a negative charge, not an unphosphorylated serine side chain. Finally, the activity of the PDPK mutants in vitro indicates that PDPK phosphorylation is not required for RNA annealing, and that such mutations do not perturb the structural competence of the protein to perform this function. Taken together, these results strongly suggest that inhibition of retrotransposition is related to a defect of phosphorylation, not to structural changes caused by replacement of an unphosphorylated serine or threonine.

The prolyl isomerase Pin1 is a critical downstream modulator of phosphorylated PDPK sites that binds phosphorylated S/T-P motifs and catalyzes *cis*/*trans* isomerization of the prolyl bond. Our finding that the serine PDPK motifs in ORF1p mediate both L1 activity and an interaction with Pin1 suggests a mechanistic role for Pin1 in L1 retrotransposition. The functional consequences of the ORF1p/Pin1 interaction could be any one of multiple known effects induced by Pin1 isomerization, including altered kinetics of phosphorylation and dephosphorylation (48, 49). Given the effects of the S18D/S27D phosphomimetics, perhaps Pin1 protects the phosphorylated state of S18 and S27, possibly by inhibiting *cis*/*trans* prolyl-sensitive phosphatases (48, 49). Although L1 activity was partially restored by S18D/S27D, Pin1 binding was not. However, Pin1 binding at these sites would not be essential for L1 activity in the context of constitutive mimicked phosphorylation if its function at S18 and S27 was to protect phosphorylation. On the other hand, it is also possible that the stringent conditions of the GST pull-down, combined with the nonquantitative nature of Western blot analysis, failed to capture a weakened interaction between Pin1 and the S18D/S27D mutant that was nonetheless sufficiently stable within the cell to permit retrotransposition, albeit at a reduced level.

Our findings that ORF1p is a substrate for protein kinases indicates that L1 has appropriated a major regulatory cascade of the host, as is the case for numerous pathogens (68). In addition to its normal and evolutionary relevant replication niche in germline and early embryonic cells (26, 27, 69–71), L1 also can be active in some somatic cells, including certain tumors and neuronal progenitor cells (19, 20, 23), as well as a consequence of aging (72). ORF1p competition for kinases in any of these cells could perturb signaling cascades. In germline and early embryonic cells, even slightly deleterious effects of this competition could provide selective pressure for adaptive evolutionary changes in components of the phosphorylation-based regulatory pathways. Although the recent attention given to potential effects of L1 on cancer progression, neuronal development, and aging have focused mainly on retrotransposition or the effects of ORF2p, increased expression of ORF1p in these cells may dramatically alter their signaling and metabolic pathways, with consequences extending far beyond those of L1-induced genetic change. Our findings thus open areas for L1 research focused not only on the interplay between ORF1p and host factors necessary for retrotransposition, but also on questions regarding the overall effects of L1 protein expression on cellular function.

Materials and Methods

Retrotransposition Assays. In this previously described tissue culture-based retrotransposition assay (9), HeLa cells were seeded in six-well plates at 2 × 10^5 cells per well, transfected with 1 μg of pRTC2-puro, and selected with G418 at 400 μg/mL beginning at 72 h posttransfection for ∼10 d before staining with KaryoMAX Giemsa (Gibco). Transfection efficiencies were assessed in parallel duplicate wells with 10 μg puromycin/mL for 24 h, starting at 1 d posttransfection, which was sufficient to kill all untransfected cells.

LC-MS/MS Analysis. ORF1p-Flag purified from HeLa cells and untagged ORF1p purified from insect cells were analyzed for phosphorylation by the Mass Spectrometry and Proteomics Resource of the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

RNA Annealing Assay. RNA annealing was measured using a FRET assay (64). Twenty nM ORF1p-Flag constructs, 100 nM Cy3-RNA, and 2× FRET buffer were incubated at 30 °C for 5 min, after which 20 μL of 100 nM Cy5-RNA was injected for final reaction concentrations of 50 nM of each RNA oligonucleotide, 10 nM ORF1p, 50 mM Tris pH 7.4, 150 mM NaCl, 3 mM $MgCl_2$, and 1 mM DTT. Cy3 was excited at 535 nm, and emissions were read at 590 nm and 680 nm every 0.7 s for 3 min.

GST Pull-Down Assay. GST or GST-Pin1 (500 nM) was immobilized on glutathione agarose before the addition of 1.5 mg of whole-cell extracts obtained from HeLa cells transfected with WT or mutant ORF1p-Flag expression plasmids. Pull-downs were washed four times, resolved via electrophoresis, transferred to nitrocellulose membranes, and probed with ANTI-FLAG M2 antibody (Sigma-Aldrich).

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