The SV40 Large T Antigen Contains a Decoy Phosphodegron That Mediates Its Interactions with Fbw7/hCdc4*

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Cell transformation by simian virus 40 (SV40) results mostly from the highly oncogenic activities of the large T antigen (LT), which corrupts the cellular checkpoint mechanisms that guard the cell cycle and the transcription, replication, and repair of DNA. The most prominent LT targets are the retinoblastoma protein (pRb) and p53. Here we report that LT binds directly to Fbw7, the substrate recognition component of the SCFFbw7 ubiquitin ligase and a human tumor suppressor. LT binding mislocalizes the nucleolar Fbw7γ isoform to the nucleoplasm. Interestingly, the binding of LT to Fbw7 occurs via a decoy phospho-degron within the C terminus of LT that closely mimics the consensus Cdc4 phospho-degron found within Fbw7 substrates. We demonstrate that, using this mode of interaction, LT can interfere with Fbw7-driven cyclin E turnover in vivo and causes increased cyclin E-associated kinase activity. Our data suggest that LT competes with cellular proteins for Fbw7 binding in a substrate-like fashion.

To utilize the host cell DNA replication machinery, small DNA tumor viruses have evolved mechanisms to override normal replication controls, such as the pRb1 pathway (1), pRb and its pocket protein family members p107 and p130 sequester E2F proteins and recruit histone deacetylases to E2F promoters to silence genes required for DNA synthesis, metabolism, and cell cycle progression (2, 3). In normal cell cycles pocket proteins are inactivated by the oscillating activities of cyclin-dependent kinases through phosphorylation.

SV40, as well as certain adenovirus and papillomavirus serotypes, produces viral oncoproteins such as the SV40 LT antigen that inactivate pRb. LT binds directly to pRb and its family members via an N-terminal LxCxE motif. This domain is normally used by D-type cyclins, histone deacetylases (HDAC1 and HDAC2), and other chromatin remodeling factors (BRG1 and BRM) for the interactions with pocket proteins and is copied by LT and other viral oncoproteins (Ad-E1A, HPV-E7).

Deletion of the LxCxE domain prevents LT from binding to pocket proteins and renders LT incompetent for cell transformation (4, 5). However, LxCxE-dependent interactions are not essential for pRb-mediated growth arrest and binding of LT via the LxCxE motif is required but not sufficient for the inactivation of pocket proteins. pRb mutants that eliminate LT binding (and that of other LxCxE-containing proteins) are still capable of repressing E2F and cause cell cycle arrest (6, 7). A second N-terminal domain was shown to cooperate with the LxCxE motif in the full inactivation of pRb family members. This region, termed the Dna4 domain, specifically binds to the Hsc70 ATPase and is required for the release of E2F from pocket proteins (8–10).

Another key cellular target of LT is the p53 tumor suppressor. The interaction of LT with p53 blocks p53-dependent growth arrest and apoptosis (11). LT contacts the DNA binding domain of p53 through a bipartite domain in its center and prevents p53 from activating transcription. Interestingly, the two p53 relatives, p63 and p73, are not targeted by LT (12–14). Besides pocket proteins and p53, a number of additional LT binding partners have been identified that are involved in the regulation of transcription (p300/CBP and p400), the spindle checkpoint (Bub1 and Bub3) (15), and DNA repair (Nbs1) (16, 17).

More recently, LT has been shown to bind to Cul7, a member of the cullin family functioning as part of E3 ubiquitin ligases in various protein turnover pathways (18). We describe here the interaction of LT with another component of the ubiquitin-machinery: the F-box protein Fbw7. Fbw7 is the substrate recognition component of the SCFFbw7 ubiquitin ligase that targets cyclin E (19–21), c-Myc (22–25), c-Jun (26, 27, 28), and Presenilin 1 (29, 30) for degradation. Fbw7 binds to its substrates through a conserved phosphorylated epitope, the Cdc4 phospho-degron or CPD (31), and Fbw7 binds to Skp1 and the remainder of the ubiquitination machinery via another domain termed the F-box. These interactions bridge the actual ubiquitin-conjugating enzyme to the substrate and lead to the ubiquitination and subsequent degradation of the substrate by the proteasome.

The Fbw7 gene is located in a chromosomal region (4q32) that is deleted in up to 30% of human tumors (32). Inactivating Fbw7 mutations occur in primary tumors and tumor cell lines (20, 21, 33). Fbw7 disruption in a genomically stable human tumor cell line leads to chromosome instability (33), and Fbw7 mutations occur in primary tumors and tumor cell lines (20, 21, 33). Fbw7 disruption in a genomically stable human tumor cell line leads to chromosome instability (33), and Fbw7 loss in mice causes early embryonic lethality (34, 35).

In this study, we identified Fbw7 as a target of LT and characterized a consensus CPD in the C terminus of LT that is regulated by phosphorylation of Thr701. However, unlike bona fide Fbw7 substrates, Fbw7 does not degrade LT. Instead, our data suggest that LT may function as a competitive inhibitor of Fbw7 function for physiologic substrates such as cyclin E.


**EXPERIMENTAL PROCEDURES**

*Cell Culture, Transfections, and Plasmids—* U2OS (human osteosarcoma), VA-13 (SV40 transformed WI-38 human lung embryonic fibroblasts, available at the ATCC), and HEK-293 (human embryonic kidney) cell lines were cultured under standard conditions in Dulbecco's modified Eagle's medium containing 10% calf serum. For transfection experiments, cells were seeded into 6-cm dishes and transfected the next day by calcium precipitation overnight at 30–40% confluence. 24 h after washing and replacing the media cells were harvested either for protein extracts or immunostaining (36). Typically, we express 1/10 of the plasmid amount of pFlag-Fbw7(3.0 μg) compared with Fbw7α and Fbw7γ (3 μg each). The in vivo Fbw7-driven cyclin E turnover assay was performed as described (37). The LT cDNA was obtained from VA-13 cells. RNA was reverse-transcribed into cDNA and the LT sequence amplified by PCR. The purified product was subcloned into an HA-tagged version of the pCS2+ vector and sequenced. VA-13 cells contain a LT sequence corresponding to the SV40 Baylor 2 strain. All point mutants and deletions were introduced by the QuikChange method (Stratagene) and confirmed by sequencing.

**Immunostaining, Immunoprecipitation, Immunoblotting, and Kinase Assays**—For immunostaining, cells were seeded and transfected on glass coverslips. Slips were fixed with ice-cold methanol:acetone (1:1) for 5 min, air-dried, and immunostained with primary antibody. After washing with phosphate-buffered saline, slips were incubated with secondary fluorescein isothiocyanate-coupled anti-mouse antibody, washed, and mounted. All protein extracts were made in 20 mM Tris pH 7.5, 100 mM NaCl, 0.5% Tween 20 lysis buffer (38). Protein expression was determined by standard procedures (SDS-PAGE, Western blot, immunoblot). Protein complexes were analyzed by immunoprecipitation as described (36). Cyclin E kinase activity was measured by immunoprecipitation kinase assays using GST-pRb as described previously (38). The following primary antibodies were used in this study: anti-FLAG (Sigma, M2), anti-HA (12CA-5), anti-LT (Santa Cruz Biotechnology, Pab 108), anti cyclin E (Santa Cruz Biotechnology, HE-111 and HE-12), and anti-Myc-tagged cyclin E (9E-10).

**RESULTS**

The Fbw7 ubiquitin ligase is produced as three alternatively spliced isoforms that localize to different subcellular compartments (32, 39). We previously reported a nucleolar isoform of Fbw7, Fbw7γ, that regulates c-Myc turnover in the nucleolus (24). While nucleolar localization of Fbw7γ was observed in various cell types, we noticed that in VA-13 cells, this isoform excludes nucleoli and exhibits nucleoplasmic staining that is similar to that observed with the Fbw7γ isoform (Fig. 1A). VA-13 cells are derived from the human diploid embryonic lung fibroblast WI-38 cell strain by SV40-mediated transformation. We thus speculated that viral transformation with SV40 caused mislocalization of Fbw7γ, perhaps by direct interaction of a viral protein with Fbw7.

We tested this hypothesis by cloning the LT cDNA from VA-13 cells and expressing it in U2OS cells, in which Fbw7γ normally localizes to the nucleolus (24). Remarkably, co-expression of LT completely eliminated Fbw7γ nucleolar localization and led to accumulation of this Fbw7 isoform in the nucleoplasm (Fig. 1B). Thus, the mislocalization of Fbw7γ in VA-13 cells is likely due to LT expression in these cells. LT contains a potent nuclear localization signal (NLS), and deletion of the NLS renders LT cytoplasmic (Fig. 1C, center panel). The LTΔNLS mutant failed to mislocalize Fbw7γ (Fig. 1B, right panel), and instead, a fraction of this mutant was detected in the nucleolus upon expression of Fbw7γ (Fig. 1C, right panel). This suggested that LTΔNLS is transported into the nucleolus via a direct interaction of LT with Fbw7γ.

Fbw7 binds to its substrates via a conserved phosphorylation epitope, the Cdc4 phospho-degron or CPD (31). The CPDs identified so far contain a central phospho-threonine followed by proline in the +1 position and preceded by hydrophobic residues in −1 and −2. Although LT could potentially interact with Fbw7 in a CPD unrelated fashion, examination of the LT primary sequence revealed a candidate CPD at the extreme C terminus with threonine 701 in its center (Fig. 2A). Interest-
Fbw7 binding (43). Thus, in analogy to cyclin E/Ser384, the interaction (37) and an S384A mutant is at least partly deficient in mediated turnover. LT/Thr 701 is a reported phosphosite. Interaction of LT with Fbw7 may require Glu 705, which produced, likely due to the cytoplasmic location of this isoform. Since the WD40 repeats are common to each of the three Fbw7 isoforms, we tested whether LT is capable of interacting with all of them. As shown in Fig. 3A each of the Fbw7 isoforms co precipitates with LT in a T701 phosphorylation-dependent manner. The interaction with Fbw7β, however, is greatly reduced, likely due to the cytoplasmic location of this isoform (Fig. 1A and Ref. 39).

Cyclin E degradation by Fbw7 requires Ser384 phosphorylation (37) and an S384A mutant is at least partly deficient in Fbw7 binding (43). Thus, in analogy to cyclin E/Ser384, the interaction of LT with Fbw7 may require Glu705, which provides a negative charge in the +4 position of Thr701 (see Fig. 2A). We tested this idea with a LT/E705A mutant in common-nucleoprecipitation assays for each of the Fbw7 isoforms. We also conclude that the interaction of LT with Fbw7 occurs in a substrate-like fashion via the WD40 repeats of Fbw7 and a classical Cdc4 phosphodegron in the C terminus of LT that is dependent on Thr 701 phosphorylation.

Since the WD40 repeats are common to each of the three Fbw7 isoforms, we tested whether LT is capable of interacting with all of them. As shown in Fig. 3A each of the Fbw7 isoforms coprecipitates with LT in a T701 phosphorylation-dependent manner. The interaction with Fbw7β, however, is greatly reduced, likely due to the cytoplasmic location of this isoform (Fig. 1A and Ref. 39).

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The data above demonstrate that LT interacts specifically with Fbw7 via a CPD in its C terminus. Because CPDs bind directly to the substrate recognition domains of Fbw7, the WD40 repeats, this interaction could potentially interfere with the turnover of Fbw7 substrates. We tested this possibility with in vivo turnover assays for Fbw7-mediated degradation of cyclin E. Cyclin E normally becomes eliminated upon coexpression of Fbw7 in a phosphorylation-dependent manner (37). We included LT in these transfection experiments and observed that LT did not interfere with Fbw7-driven cyclin E turnover when large amounts of Fbw7 were expressed. However, when we reduced the expression of Fbw7 by titration, LT impaired cyclin E degradation in a T701 phosphorylation-dependent manner (Fig. 4A). These latter conditions are more likely to mimic the stoichiometric relationships between LT and Fbw7 in cells infected with SV40, which contain high levels of LT. Moreover, we observed increased cyclin E abundance caused by LT, but not the LT/T701A mutant, even in the absence of Fbw7 cotransfection, suggesting interference with the endogenous Fbw7 pathway (compare lanes 2 and 3 in Fig. 4, A and B). To test this directly we compared wild-type cyclin E with an Fbw7-insensitive cyclin E/T380A mutant that has a threonine 380 to alanine substitution. This mutant displays increased stability and could not be further stabilized by LT (Fig. 4C). Therefore, the phosphorylation-specific LT-mediated increase of cyclin E is LT701-dependent. Because Fbw7 specifically targets the catalytically active fraction of cyclin E, we determined whether LT expression altered endogenous cyclin E kinase activity. We found that LT expression consistently increased endogenous cyclin E kinase activity greater than that seen with LT/T701A (2.8-fold versus 1.8-fold, Fig. 4D) and this supports the idea that whereas both LT proteins increase endogenous cyclin E transcription (via E2F), only wild-type LT reduces cyclin E turnover. These data underestimate the total effect of LT expression on endogenous cyclin E activity by at least 2-fold, because the transient transfection efficiency of U2OS cells in these experiments is less than 50% (data not shown).

**DISCUSSION**

The SV40 LT protein binds to a number of host cell proteins and disrupts their normal functions. LT has thus proven to be a useful tool for the identification of important cancer-associated proteins, including pRB family members and p53. Our finding that LT binds directly to Fbw7 fits well with the general function of LT to specifically inactivate tumor suppressor genes. In fact, Fbw7 seems an ideal prey for LT, since almost every Fbw7 substrate is a potent oncogene (cyclin E, c-Myc, c-Jun, Notch), and Fbw7 inactivation would stabilize these substrates. Interestingly, in addition to being Fbw7 substrates, both cyclin E (via E2F) and Notch (45) are transcriptionally activated by LT, suggesting that LT deregulates these proteins through both transcriptional and post-transcriptional mechanisms. In fact, because Fbw7 targets catalytically active cyclin E, disruption of the Fbw7 pathway is required for overexpressed cyclin E to be maximally active (46). LT expression
FIG. 3. LT interacts with Fbw7 in a substrate-like mode. A, LT binds to each Fbw7 isoform in a LT/T701-dependent manner. 293 cells were transfected as indicated and anti-HA immunoprecipitates (anti-LT) blotted for Fbw7-binding (anti-FLAG). B, the negative charge in the +4 position (LT/ Glu705) has an essential role in Fbw7 binding. 293 cells were transfected as indicated and processed identical as in A. The FLAG immunoblots were subsequently probed with LT antibody (+LT). The asterisk marks the heavy chain signal from the anti-HA immunoprecipitation.

Fig. 4. LT interferes with Fbw7 substrate recognition. A, LT partly prevents Fbw7-mediated cyclin E turnover. U2OS cells were transfected and cell lysates immunoblotted as indicated. 300 ng of Myc-tagged cyclin E plasmid were cotransfected with 4 μg of each Cdk2 and LT (or LT/T701A). Fbw7a amounts are in micrograms of plasmid DNA transfected into 6-cm dishes. The asterisks mark nonspecific cross-reactions of the used antibodies and serve as loading control. B, increased cyclin E levels in 293 cells upon LT expression. 293 cells were cotransfected with 300 ng of Myc-tagged cyclin E plasmid and either empty vector or LT and Cdk2 (3 μg each) and cell lysates immunoblotted for both proteins. C, a cyclin E/T380A mutant is insensitive to LT expression. 293 cells were transfected as in B, including Cdk2, and lysates immunoblotted as indicated. D, LT stimulates endogenous cyclin E-associated kinase activity in a Thr701-phosphorylation-dependent manner. U2OS cells were transfected with either LT or the LT/T701A mutant and lysates immunoblotted as indicated (top and middle panels) or anti-cyclin E immunoprecipitates subjected to kinase reactions using GST-pRb as substrate (bottom panel). The increase of cyclin E-associated kinase activity was consistently observed in five independent experiments and represents an underestimate due to the transfection efficiency of U2OS cells (less than 50%).

may thus increase cyclin E activity because it targets both its synthesis and destruction pathways.

We have shown that the Fbw7γ isoform fails to localize to the nucleolus in SV40 transformed VA-13 cells (Fig. 1). Our finding that LT is responsible for this mislocalization also explains the nucleoplasmic localization of Fbw7γ in 293T cells, which also express LT (43). The mislocalization of Fbw7γ by LT may lead to the deregulated activity of Fbw7γ targets within the nucleolus, including c-Myc (24). However, thus far we have not observed that LT activates the growth-promoting activity of c-Myc (a nuclear c-Myc function) or causes c-Myc nucleolar accumulation. This may reflect the pleiotropic activities of LT expression, or perhaps, because LT has many “Myc-like” activities, these pathways may already be maximally active in LT expressing cells.

Previous work has shown that the N terminus of LT is sufficient to transform mouse cells (47). We thus considered it unlikely that binding to Fbw7 was required for LT-mediated cell transformation, and instead speculated that it may enhance the oncogenic potency of LT under certain conditions. However, we have not observed any Thr701-dependent effects in cotransformation assays using LT and Ras expression in primary mouse embryo fibroblasts (data not shown). We also note that the LT CPD is contained within the variable domain in the LT C terminus (48), which is characterized by its high degree of primary sequence flexibility. In fact, a small number of SV40 isolates from SV40-induced tumors do not contain the Thr701 phosphorylation site. While it is possible that these strains mutated LT subsequent to the establishment of tumors, this is also consistent with the idea that binding to Fbw7 is not essential for LT function. Moreover, we did not detect candidate CPDs in the HPV-E6/E7 and Ad-E1A/E1B oncoproteins, indicating that Fbw7 binding is not essential for oncogenesis by other small DNA tumor viruses.

Surprisingly, we found that LT binds to Fbw7 in a substrate-like fashion via a decoy CPD. The exquisite conformation of the LT binding site to consensus bona fide Fbw7 substrates suggests that this mode of binding is physiologically important. One possibility is that this binding mode allows Fbw7 to remain active as a ubiquitin ligase, since other SCF components can still be recruited to the Fbw7 F-box. Analogous to the HPV-E6 oncoprotein that bridges the E6AP HECT domain ubiquitin ligase to p53, this would potentially allow LT to make use of the SCF<sup>Fbw7</sup> ubiquitin ligase to degrade a protein(s) that normally is not a substrate of Fbw7. Alternatively, this binding mode might allow LT to specifically inhibit Fbw7 function, since other SCF complexes would presumably be unaffected by LT. Additional studies are needed to further define the role of Fbw7 in regulating LT function in cellular transformation and SV40 replication.

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