Crystal structure of SV40 large T-antigen bound to p53: interplay between a viral oncoprotein and a cellular tumor suppressor

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The transformation potential of Simian Virus 40 depends on the activities of large T-antigen (LTag), which interacts with several cellular tumor suppressors including the important “guardian” of the genome, p53. Inhibition of p53 function by LTag is necessary for both efficient viral replication and cellular transformation. We determined the crystal structure of LTag in complex with p53. The structure reveals an unexpected hexameric complex of LTag binding six p53 monomers. Structure-guided mutagenesis of LTag and p53 residues supported the p53–LTag interface defined by the complex structure. The structure also shows that LTag binding induces dramatic conformational changes at the DNA-binding area of p53, which is achieved partially through an unusual “methionine switch” within p53. In the complex structure, LTag occupies the whole p53 DNA-binding surface and likely interferes with formation of a functional p53 tetramer. In addition, we showed that p53 inhibited LTag helicase function through direct complex formation.

[**Keywords:** Cell transformation; cell growth control; tumor suppressor; viral oncoprotein]

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Large T-antigen (LTag), a viral oncoprotein, is a major early gene product encoded by SV40. LTag has multiple diverse biological functions. LTag is a member of the helicase family III (part of the AAA⁺ superfamily) and functions for viral DNA replication (Li and Kelly 1984; Neuwald et al. 1999). On the other hand, LTag can differentiate and transform cells into continuously dividing cells upon viral infection (Endo and Nadal-Ginard 1998; Endo 2004). When expressed on its own, LTag is also able to induce proliferation of quiescent cells, transform several cell types, and induce tumor formation in experimental animals (Fanning and Knippers 1992). LTag is thought to overcome cell cycle checkpoints by hindering the function of several important cellular tumor suppressors including p53 (Srinivasan et al. 1997; Pipas 1998; DeCaprio 1999; Kim et al. 2001).

p53 was first identified as a cellular protein interacting with LTag (Lane and Crawford 1979; Crawford et al. 1980), and subsequently its critical role in regulating cell growth and maintaining genomic stability was estab-

lished (Lane 1992; Levine 1997; Zhao et al. 2000). The p53 gene is found mutated in >50% of human cancers, indicating that this protein governs important pathways that suppress tumor cell growth (Hollstein et al. 1991). Among the most important roles p53 plays is its ability to either arrest the cell cycle and allow for DNA repair or fatefully progress toward apoptotic programmed cell death. p53 is known to be a transcription factor regulating >160 genes (Zhao et al. 2000). In addition to controlling responses to DNA damage, p53 plays a critical role in defending against viral infection by triggering cell death in infected cells (Nakamura et al. 2001; Munoz-Fontela et al. 2005).

The importance of p53 is also highlighted by the fact that many different viral oncoproteins target and inhibit p53 function in order to deregulate cellular growth control. In addition to SV40 LTag, other viral oncoproteins known to interact and inhibit p53 include Adenovirus E1B, Hepatitis B virus protein X, Papilloma virus E6, Epstein-Barr virus EBNA-5, and the human T-cell lymphotropic virus tax protein (Szekely et al. 1993; Wang et al. 1994; Pise-Masison et al. 1998; Nevins 2001). Interestingly, even though other viral oncoproteins, such as the papillomavirus E6, are known to target p53 for

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degradation, expression of LTag stabilizes cellular p53 (increasing the half-life of the protein) while inhibiting its transactivation activity (Deppert et al. 1989; Pipas and Levine 2001). These results imply that LTag inactivates p53 by a distinct mechanism unrelated to the classic pathways involving down-regulation of protein levels by cellular processes triggered by post-translational modifications.

Here we present the 3.16 Å crystal structure of the helicase domain of LTag bound to the DNA-binding domain of human p53, which represents the first structure of a viral oncoprotein bound to the p53 tumor suppressor. This structure identifies the molecular interactions between LTag and p53. The structure also reveals a dramatic local conformational change of p53 on its DNA-binding loop upon interaction with LTag. Significantly, LTag occupies a significant portion of the DNA-binding surface of p53 as well as a region that otherwise would be involved in assembly of the tetrameric p53. In addition, we demonstrated that p53 inhibited the helicase function of LTag through complex formation, suggesting a possible interplay between the viral oncoprotein and p53 tumor suppressor.

Results

Overall structure of the complex

To study the interaction between LTag and p53, we purified a complex containing the helicase domain (residues 251–627) of LTag (Li et al. 2003) and the DNA-binding domain (residues 92–292) of human p53. The LTag and p53 protein complex was analyzed using size exclusion chromatography. We observed three elution peaks (peak 1, 2, 3) in the size exclusion profile (Fig. 1A), which correspond to the expected size for a hexameric LTag–p53 complex in peak 1, a monomeric LTag–p53 in peak 2, and a monomeric p53 in peak 3. The LTag–p53 complex from peak 1 crystallized in a space group of $p2_1$ with unit cell dimensions of $a = 127.0$ Å, $b = 182.7$ Å, $c = 262.1$ Å, $\alpha = 90.0^\circ$, $\beta = 92.0^\circ$, $\gamma = 90.0^\circ$ (Fig. 1; Table 1).

The LTag–p53 complex is composed of one LTag hexamer with each individual LTag molecule binding one monomeric p53 (one LTag hexamer:six p53s) (Figs. 1C–F). The structure contained 12 molecules of both p53 and LTag (24 molecules total) in one asymmetric unit, which are arranged in two copies of the hexameric complex (Fig. 1C). Previously, we observed a conformation in LTag that was thought to be entirely dependent on ATP binding to the helicase domain of the LTag. Interestingly, in the LTag–p53 complex, the LTag hexamer assumes the conformation nearly identical to the LTag–ATP crystal structure (RMSD = 0.797 over 17,604 atoms) (Gai et al. 2004), although nucleotides were absent from the crystallization solution of this complex. The p53 DNA-binding domain in the LTag–p53 complex has an overall fold similar to the previously reported structure, known as a β -sandwich scaffold, with two large loops (L2 and L3) and two α -helices (h1 and h2) (Fig. 1B; Cho et al. 1994; Gorina and Pavletich 1996; Joo et al. 2002). In the

LTag–p53 complex, each p53 binds to the α -helical portion of LTag that is located on the outer side of the LTag hexamer (domain D3) (Figs. 1B–F). Binding of six p53 molecules on an LTag hexamer extends the outward projection of the six corners of the hexamer, enhancing a propeller-like appearance of the structure (Fig. 1C). The p53-binding site is adjacent to the postulated single-stranded DNA (ssDNA) exit side channel on the surface of LTag (Li et al. 2003), a location that is also proximal to the proposed RPA-binding site of LTag (Weissbart et al. 1998).

The interface between individual LTag and p53 is relatively large (buries ~ 1661 Å²). A total of 23 LTag residues and 19 p53 residues are either buried in this interface or are found to directly participate in the interactions between these two molecules. The interacting residues of LTag are located on α -helices h8, h14, h15, and h16 and the surrounding loop region (see Fig. 1B). The portions of p53 that interact with LTag contain α -helix 1 (h1), α -helix 2 (h2), and Loop 3 (L3) (Fig. 1B). The residues on the surface of p53 that interact with LTag are conserved from mice to humans.

Interactions at LTag–p53 region 1 and the p53 zinc motif

The LTag–p53 interface can be arbitrarily divided into two regions (see boxes 1 and 2 in Fig. 2A), which are described in detail below. Within region 1 (Fig. 2A), three residues, P177, H178, and R181, extend from α -helix h1 of p53 and make direct contact with α -helix h15 of LTag (Fig. 2B). Interestingly, the p53 residues that interact with LTag within this region 1 are also located within the interface between two p53 molecules (Cho et al. 1994; Ho et al. 2006). Molecular dynamic simulations and sequence analysis also suggest that the residues within the surface surrounding region 1 may play a significant role in stabilizing the p53 tetramer when it is bound to DNA (Ma et al. 2005), suggesting that LTag binding may interfere with tetramerization of p53.

It is notable that within the LTag–p53 interface between regions 1 and 2 on p53 there exists a Zn-binding motif (Fig. 2C). The p53 Zn atom is coordinated by residues C176, H179, C238, and C242 that are located on loops L2 and L3, respectively. It is conceivable that the zinc plays a role of stabilizing loops L2 and L3 through coordination, which may contribute to the stable interaction with LTag. This stabilization of two loops by Zn coordination is also seen at the protein–protein interface of an archaeal MCM complex (Fletcher et al. 2003).

Interactions within LTag–p53 region 2

A more dense network of interactions are found in region 2, which are shown in Figure 2, A, D, and E. Region 2 contains 16 residues from p53 that make bonding contacts with amino acids on the surface of LTag. Most of the residues involved in the interactions are located on h2 and L3 of p53, and on h15 and h16 of LTag (Fig. 2D,E).

Figure 1. Overall structure of LTag-p53 complex. (A) The gel filtration profile of the LTag-p53 complex. The protein complex was stable when analyzed by size-exclusion chromatography on a superdex200 column. The positions for the molecular weight standard markers are indicated. Peak 1 contained LTag-p53 complex in a hexameric form (~360 kDa), peak 2 contained LTag-p53 complex in a monomeric form (~69 kDa), and peak 3 contained p53 alone (~22 kDa). The three SDS-PAGE gels below the profile show the presence of LTag and p53 in the three peaks. The excess of p53 was eluted in peak 3. (B) The monomer structure of LTag (in green) and p53 (in cyan). In order to better display the interface, each molecule was rotated 60° from its original position within the complex. The regions involved in the interface contacts in the complex are colored in yellow. LTag domains D1, D2, and D3 are indicated. (C,D) The top views of the LTag hexamer in complex with p53 (C) and the LTag hexamer stripping off p53 (D). Each of the six subunits of a LTag hexamer binds one p53 molecule as shown in A. (E,F) The side views of the LTag hexamer in complex with p53 (E) and the hexamer stripping of the p53 (F). The side view of the LTag-p53 complex structure reveals that p53 binds to the larger C-terminal tier of LTag.

These residues contain both hydrophilic and hydrophobic side chains, and form a diverse set of strong interactions. Of significance, p53 residue R248 on loop L3 interacts with LTag D402 (Fig. 2D). R248 of p53 is known to be the most frequently mutated residue in human cancers (Hollstein et al. 1991) and plays a critical role in p53's ability to interact with DNA (Cho et al. 1994). This R248 of p53 also plays a critical role in the recognition by LTag.

Besides the polar interactions described above, the LTag surface has a hydrophobic pocket within the central area of region 2. This pocket is composed of residues L609, Y612, W581, and Y582 (Fig. 2D). This pocket in LTag is occupied by the p53 residue M246 extending from the L3 loop of p53 (Figs. 2D, 3D), forming a strong

hydrophobic interaction that is expected to play an important role in the binding of p53 by LTag.

The conformational change of p53 upon LTag binding

Surprisingly, the p53 structure at the LTag-binding surface is different from that seen in the DNA-binding state of p53 (Cho et al. 1994). In particular, the L3 loop of p53 in the DNA-binding state packs tightly with its interior hydrophobic core (Fig. 3A; Cho et al. 1994). However, when binding to LTag, the L3 loop undergoes a dramatic conformational change: It extends away from the core p53 (Fig. 3B,C), while the side chain of M246 on L3 flips out of the interior core of p53 to protrude into the hydrophobic pocket of LTag (Fig. 3C,D), establishing an

Table 1. Crystallographic data (native) and refinement statistics

Space group	$p2_1$ $a = 127.0 \text{ \AA}$, $b = 182.7 \text{ \AA}$, $c = 262.0 \text{ \AA}$ $\alpha = 90.0^\circ$, $\beta = 92.0^\circ$, $\gamma = 90.0^\circ$
Resolution ($\text{\AA}$)	20–3.16
Observations	2,092,304
Unique observations	204,669
Completeness (%) (last bin)	97.5 (94.3)
Number of molecules/asymmetric unit	24
R_{sym} (%) (last bin)	0.069 (0.398)
I/S (last bin)	30.6 (4.5)
Refinement statistics	
R_{work}	26.14%
R_{free}	30.81%
Total protein atoms	53,932
RMS deviation	
Bonds ($\text{\AA}$)	0.00127
Angles ($^\circ$)	1.212

intimate interaction in the central area of region 2. This flip-out action of M246 generates a void in the protein hydrophobic core of p53, which is filled by another residue, M243, that flips inside from its originally exposed position on the protein surface (Fig. 3C). Furthermore, R248 on the L3 loop is repositioned nearly 9 $\text{\AA}$ to establish contact with LTag residue D402 (shown in Figs. 2D, 3D). Thus, by inducing the dramatic conformational switch of the p53 L3-loop region, the LTag oncoprotein establishes intimate interactions with the p53 tumor suppressor.

The DNA-binding and LTag-binding surfaces of p53

In the crystal structure of the p53–DNA complex, the L3 loop of p53 makes several important contacts with DNA (Cho et al. 1994). Thus, the L3 loop of p53 is an overlap-

ping site for binding both DNA and LTag, only in different conformational states. However, the buried surface area of p53 at the p53–LTag interface is approximately three times greater than that at the p53–DNA interface. Six of the eight p53 residues contacting DNA (Cho et al. 1994) are completely buried at the p53–LTag interface (Fig. 4A). Indeed, three of the most frequently mutated p53 residues in human cancer (G245, R248, R273) are all within region 2 of the LTag–p53 interface (locations are indicated by stars [*] in Fig. 4A). Therefore, LTag binding to p53 will likely block the functions of these residues of p53 by covering the entire DNA-binding surface of p53.

Mutational analysis of the LTag–p53 interactions

We tested the relevance of the observed binding interface of LTag and p53 through structure-guided mutagenesis of both LTag and p53. On the LTag side, residues V585 and D604 point their side chains toward p53 (Fig. 4B). Therefore, we created V585R and D604R mutations to generate longer R side chains to present steric hindrance for p53 binding. On the p53 side, mutant M246R was generated to test its role within the L3 loop in the interaction with the hydrophobic pocket of LTag (Figs. 3D, 4B). The binding assays through glutathione-S-transferase (GST)-pull-down showed that all designed mutants, either LTag V585R and D604R or p53 M246R, disrupted the LTag–p53 complex formation (Fig. 4C). A similar result was obtained when assayed by gel filtration chromatography, which showed that the mutant proteins were unable to form a stable complex (Fig. 4D). Thus, mutations on either side of the structural interface can disrupt LTag–p53 complex formation.

p53 binding and LTag helicase activity

p53 is reported to inhibit the replication of the viral origin DNA in cell extract containing LTag (Miller et al.

Figure 2. Residues involved in LTag–p53 interactions. (A) A ribbon illustration of a monomeric LTag–p53 complex taken from the hexameric structure. The boxes indicate the two regions at the interface between p53 (in yellow) and LTag (in green). (B) The close-up view of the interactions in box 1, showing that p53 residues P177 and H178 make van der Waals contacts with LTag residues A586 and V597, respectively, and p53 R181 bonds with LTag Q593. (C) The LTag–p53 interface around the zinc motif of p53. The zinc atom is between the two boxed regions, bridging and stabilizing p53 surface loops that contact LTag. (D,E) The close-up views of the detailed interactions within box 2. D shows a hydrophobic pocket formed by LTag residues L609, W581, Y582, and Y612, with which p53 M246 interacts. E shows that four bonds form between p53 N288, R273, N239, and R280 with LTag residues Q613, D604, E601, and D402, respectively.

Figure 3. The conformational change of p53 upon LTag binding. (A,B) The L3 conformation of p53 in the DNA-binding or LTag-binding state. (C) Superposition of the p53 Ca carbon, showing the L3 conformation changes upon LTag binding. The LTag-binding state of p53 is shown with a yellow backbone and thick side chains, and the DNA-binding state with a light-blue backbone and thin side chain. Arrows indicate the movement of the three key residues upon LTag binding. The originally buried M246 residue in the DNA-binding state flips out from the interior core to extend and insert its side chain into the hydrophobic pocket on LTag as shown in D. At the same time, the void left by the flipped M246 is filled by another residue, M243, that is flipped inside from its exposed position in the DNA-binding state. This “methionine switch” allows L3 to have a large conformational change. (D) Interactions of p53 with LTag through the L3 loop. The flipped-out M246 on the L3 loop extends and inserts into the hydrophobic pocket on LTag, while R248 establishes a salt bridge on the surface (also see Fig. 2D). (E,F) The 12-fold averaged electron density map and the simulated annealing omit map at the position of the L3 loop of p53 in the LTag complex structure.

1995). Such inhibition could be due to the direct binding to DNA as a “roadblock” to sterically inhibit passage of the LTag helicase as it travels along the DNA. Alternatively, the inhibition of the helicase activity could be through physical interaction with LTag. We reasoned that LTag mutants with defective p53 binding might be useful in providing direct evidence regarding which of these mechanisms described above applies to p53 functions in viral replication. We examined the effect of the wild-type p53 protein (DNA-binding domain) on the helicase activity of the wild type as well as the two mutant LTag constructs that are defective in p53 binding (Fig. 5). p53 showed significant inhibitory effect on the helicase activity of the wild-type LTag (Fig. 5A), but has no detectable effect on the helicase activity of the two LTag mutants (V585R, D604R) that are defective in p53 binding (Fig. 5A,B). These results indicate that p53 inhibits LTag helicase activity through a direct contact, which involves the interface observed in our LTag–p53 complex.

Discussion

Many tumor viruses target p53 for inhibition as an important mechanism to overtake cellular growth control, illustrating the critical role of p53 in maintaining the normal cell division cycle. In this study, we describe the crystal structure of the protein complex of SV40 LTag

oncoprotein and p53 tumor suppressor. This structure presents the detailed bonding interactions between LTag and p53 and provides a molecular explanation for how LTag inhibits p53 function through specific protein–protein interactions. In addition, the crystal structure also confirms many prior hypotheses and biochemical observations about the LTag–p53 interactions that play an important role in cell transformation resulting from the virus infection.

Mapping of mutational residues to the interface

Previous mutagenic studies have identified many mutations that affect LTag transformation efficiency (Lin and Simmons 1991; Peden et al. 1998). Four of these mutated residues, P399, D402, C411, and P584, are located on the surface of LTag that is around the interface for p53 binding (Fig. 6A). Of these four residues, D402 and P584 directly interact with p53 in the complex structure. D402 forms a salt bridge with p53 residue R248, which is one of the most frequently mutated residues in cancers, while P584 packs with the main chain of p53 around M243 (Fig. 6B).

Methionine switch of p53

There are unique features at the LTag–p53 interface that differentiate the p53 structure in the LTag-bound state

Figure 4. Mutational analysis at the LTag-p53 interface. (A) The surface representation of p53, showing locations of the DNA-binding and LTag-binding residues. The DNA-binding residues are colored in red, the LTag-binding residues in blue, and residues involved in both DNA binding and LTag binding are colored in silver. The figure shows that p53-DNA interaction residues are adjacent and overlapping with the p53-LTag interface. The binding of LTag to these p53 residues can effectively shield the entire DNA-binding surface of p53, which seems a very reliable mechanism for inhibiting transactivation at the p53-dependent promoters. Interestingly, the three most commonly mutated p53 residues in cancer—R273, R248, and G245 (indicated by *)—are also recognized by LTag. (B) The positions of the three residues at the LTag-p53 interface; that is, LTag residues V585 and D604 (in cyan) and p53 residue M246 (in orange), which are targeted for mutational analysis to verify the structure. The structure shows that these residues contribute significantly to the binding of LTag and p53, and thus mutations of these residues are expected to disrupt LTag-p53 interactions. (C) Formation of LTag-p53 complex using the LTag or p53 mutants, as assayed by the GST pull-down method. GST-LT fusion proteins (both wild-type and mutant LTag) bound on the glutathione resin were used to test its ability to bind to wild-type and mutant p53 proteins on the column. Lanes 1 and 2 are controls for nonspecific binding of wild-type (wt) or mutant p53 proteins alone to the glutathione resin, respectively, lane 3 is the wild-type GST-LT pulling down wild-type p53 on the glutathione resin, lane 4 is the wild-type GST-LT pulling down mutant p53 M246R (M/R), lane 5 is the mutant GST-LT V585R (V/R) pulling down wild-type p53, and lane 6 is the mutant GST-LT D604R (D/R) pulling down wild-type p53. The result shows that LTag mutants V585R and D604R and the p53 mutant M246R all disrupted the LTag-p53 complex formation. (D) Effects of structure-based mutations on LTag-p53 complex formation, as assayed by size-exclusion chromatography. The various mutants and wild-type protein mixtures of LTag and p53 were incubated for 30 min at 25°C prior to loading on a superdex200 analytical gel filtration column. The positions of the molecular weight markers are indicated on the chromatograph. Fractions corresponding to the three elution peaks (peaks 1, 2, and 3) for each of the experiments were analyzed using SDS-PAGE; the results are shown in the panel below the gel filtration chromatograph. The elution volumes equivalent to peaks 1 and 2, where the hexameric and nonhexameric complexes of LTag-p53 normally migrate, contained very little p53 when either mutant LTag or mutant p53 was used to form the complex with the corresponding wild-type partner. Both the affinity-binding studies (shown in C) and the gel filtration analyses indicated that the structure-based mutations have the desired effect disrupting the interactions between LTag and p53.

from the p53 structures determined previously by others (Cho et al. 1994; Gorina and Pavletich 1996; Derbyshire et al. 2002; Joo et al. 2002). Most notably, the conformational change in L3 provides a unique platform for interactions at the LTag-p53 interface. Several important features are built into L3 that allow the conformational change to take place. Firstly, the position of the two

methionines within the loop allows for one methionine to participate in hydrophobic packing with the interior core, while the other is exposed to interact with a binding partner. Secondly, while the packing of M243 or M246 within the p53 core helps stabilize the L3 loop, the packing has to be weak enough to allow the "methionine switch" to occur, which presumably takes place upon

Figure 5. Effect of p53 binding on LTag helicase activity. (A) The helicase activity of wild-type (wt) and mutant LTag was assayed in the presence of varying amounts of p53. The LTag mutants V585R and D604R used in this assay are defective in binding to p53. For each gel on this panel, lanes 1 and 2 are the boiled and unboiled dsDNA substrate alone. Lanes 3–7 contain 20 nM wild-type or mutant LTag proteins. The p53 concentrations are as follows: 0 nM (lanes 1–3); 12.5 nM (lane 4); 25 nM (lane 5); 50 nM (lane 6); 75 nM (lane 7). (B) A plot summarizing the results of the helicase assays shown in A. The helicase activity of wild-type LTag was proportionally inhibited by higher concentrations of p53. In contrast, the two LTag mutants that are defective in p53 binding were insensitive to even the highest concentrations of p53 tested.

Figure 6. Mapping of LTag mutations defective in transformation and p53 binding. (A) Mapping of LTag mutations that disrupt transformation (colored in blue and purple) on the LTag surface. These residues are located near or within the p53-binding surface (colored in yellow). (B) Interactions with p53 (in yellow and orange) through two of the LTag residues (D402 and P584, in green and cyan) critical for cell transformation by LTag. D402 is involved in a salt bridge with R248 of p53; residue P584 makes van der Waals contact with M243 of p53. (C,D) DNA mimicry by LTag in one of the interface areas, where p53 (in yellow) uses the same set of side chains (mostly from h2, residues R273, R280, N288, and N239) to bond with DNA in C or LTag in D. (E) Surface rendering of p53 (in yellow) and LTag (in green) complex, showing the bound p53 is adjacent to LTag's side-channel opening (in blue).

LTag-p53 binding. Thirdly, on the space between M243 and M246 are two consecutive glycines that provide the necessary flexibility for the methionine switch as well as for the movement of R248 to occur in order to achieve the optimal interaction with LTag.

The binding interface of p53 with other proteins

The LTag-binding interface covers the regions around L3, L2, h1, and h2 of p53, shielding the entire DNA-binding surface of p53. Two previous p53-protein crystal structures reveal that 53bp1 or 53bp2 also establishes numerous interactions with L3 and residues within and around p53 region 1 (Gorina and Pavletich 1996; Derbyshire et al. 2002; Joo et al. 2002). However, neither protein establishes significant contacts with h2 of p53. Because of this, one model proposes that p53 in the complex with 53bp1 may still have nonspecific DNA-binding activity, thus allowing the proteins to function together at the sites of DNA damage (Gorina and Pavletich 1996; Joo et al. 2002). A similar mechanism was proposed for the 53bp2-p53 complex, which is known to specifically transactivate apoptotic-specific genes (Samuels-Lev et al. 2001). These hypotheses are consistent with the finding that 53bp1 and 53bp2 regulate p53 transactivation activity in response to stress (Samuels-Lev et al. 2001; Bergamaschi et al. 2004). In contrast to these cellular p53 cofactors, the LTag oncoprotein may need to inhibit p53 transactivation function to support

viral replication (Pipas and Levine 2001); this may be achieved efficiently by sequestration or the entire p53 DNA-binding surface.

A DNA-mimicry and p53 dimerization interface

Interestingly, p53 uses the same set of charged residues on h2 to interact with both DNA and LTag through charge-charge and hydrogen-bond interaction (Fig. 6C,D). From this sense, the interaction of p53 h2 and LTag can be considered a form of DNA mimicry, even though the exact bonds are not duplicated. In addition to the interactions described in the DNA mimicry, LTag partially buries a postulated p53 core domain dimerization site located adjacently to p53 h1 (Ma et al. 2005; Veprintsev et al. 2006). This p53 dimerization region may play a role in p53 tetramerization and stabilize its association with DNA; thus LTag may also inhibit p53 tetramerization through direct binding.

Reciprocal inhibition between LTag and p53

While LTag can inhibit p53 function, p53 has also been previously shown to inhibit viral DNA replication (Bargonetti et al. 1992; Miller et al. 1995). This two-way inhibition may be the result of interplay between viral infection and host-defense. On one hand, the virus needs to inhibit p53 transactivation activity to promote cell

cycle progression and allow viral DNA replication (Pipas and Levine 2001). Physical association of LTag with p53 obviously plays a critical role in the inhibition of p53 function. On the other hand, p53 may also inhibit LTag helicase function through physical association (Fig. 5), which could slow down viral replication at the beginning of viral infection. Because the p53-binding site is right next to the side channel proposed for ssDNA exit (Fig. 6E), p53 can also function as a blockage of the side channel in DNA unwinding. Moreover, binding of p53 to the LTag helicase domain could sterically inhibit the loading of other replication proteins to LTag, including DNA polymerase, primase, topoisomerase I, and RPA (Ott et al. 2002; Arunkumar et al. 2005), which may further inhibit viral DNA replication. This inhibition of viral replication, which may be a form of innate antiviral activity of p53 in addition to its proapoptotic activity, will be overcome when the expression level of LTag far exceeds that of p53.

We have described the crystal structure of the complex containing SV40 LTag oncoprotein and p53 tumor suppressor. Multiple structure-based mutagenesis and functional studies have confirmed the observed interface between p53 and LTag in the complex structure. These results have provided insights into a detailed molecular mechanism by which a viral oncoprotein can target key functional surfaces of p53 to block its tumor suppressor activity.

Materials and methods

Protein chemistry and crystallization

The GST fusion system was used to affinity-purify the LTag helicase domain (residues 251–627) from *Escherichia coli*, as described previously (Li et al. 2003). Briefly, the protein was expressed as a GST-LT fusion using the pGEX-2T vector, with a thrombin cleavage site between GST and LTag. The fusion protein was first purified by glutathione affinity column. The GST was released by thrombin cleavage, and the LTag fragment was further purified by ion exchange and superdex200 gel filtration chromatography. Recombinant human p53 containing residues 92–292 was expressed in *E. coli* and purified in a manner similar to that described for LTag.

Purified LTag and p53 proteins (15 mg/mL) were mixed at an approximate ratio of 1:1.2 (LTag:p53) and crystallized by hanging-drop vapor diffusion at 25°C using 70 mM MOPS (pH 6.5), 5% PEG 4000, 9% glycerol, and 22 mM LiSO₄. The diffraction of the crystals was improved from ~4 Å to 3.1 Å through dehydration by placing the crystals in the hanging drop over a well containing 70 mM MOPS (pH 6.5), 5% PEG 4000, 35% glycerol, and 22 mM LiSO₄ for 12 h prior to freezing the crystals by submersion in liquid nitrogen.

Data collection, structure determination, and refinement

Native and Se-SAD data sets were collected at the APS 19ID (SBC) and 14BMC (Bio-CARS) beamlines, Argonne National Laboratory. Data were processed with HKL2000 (Otwinowski and Minor 1997). To solve the structure, various combinations of LTag oligomers were used as a search model for molecular replacement (MR) using MOLREP implemented in CCP4 (Collaborative Computational Project, Number 4 1994; Vagin and

Teplyakov 2000). Only the hexamer in the ATP-bound form (PDB ID 1SVM) yielded a correct solution, with 12 LTag molecules in the form of two hexamers per asymmetric unit. After the initial MR replacement solution was determined, rigid body and simulated annealing refinements were performed using CNS (Brunger et al. 2001). After an initial round of refinement, the phases of the partial model were of sufficient quality to reveal clearly defined electron density for the 12 missing p53 molecules surrounding the two LTag hexamers. Phases from MR were used to calculate an anomalous difference map using a SAD data set collected on LTag-p53 crystals grown with Se-Met-substituted p53, which revealed the positions of all the Se-Met residues of the 12 p53 molecules, as well as the Zn atoms in both LTag hexamers and additional Zn atoms located in the p53s. The location of the Se and Zn atoms greatly facilitated the model building of p53 by serving as definitive landmarks in the electron density map. Twelvelfold NCS restraints were applied during refinements of the p53 domains. The final refinement statistics and geometry as defined by procheck were good (see Table 1).

Helicase assay

After purification, a 60-nucleotide (nt) primer, 5'-TTTTTTTTT TTTTTTTTTTTTTTTCGCGCGGGGAGAGCGGTTTGC GTATTGGGCGCC-3' (Operon), was labeled with [γ -³²P]ATP and then annealed to M13mp18 (NEB) circular ssDNA in a 2:1 ratio in 300 mM NaCl and 20 mM Tris (pH 8.0), resulting in a 34-base-pair (bp) double-stranded DNA (dsDNA) region and a 26-nt 3' overhang. The dsDNA substrate was then purified using 700 μ L of Sephacryl 300HR resin in a 2-mL glass column (Bio-Rad). Twenty-microliter reactions containing 20 mM Tris (pH 7.8), 10 mM MgCl₂, 1 mM DTT, 5 mM ATP, 0.1 mg/mL BSA, 5 fmol of dsDNA substrate, LTag, and p53 proteins were assembled on ice and then incubated at 37°C for 60 min, followed by the addition of 5 $\times$ stop solution (10 mM EDTA, 0.5% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue, and 50% glycerol). The reactions were electrophoresed for 50 min at 150 V on a 12% acrylamide gel in 1 $\times$ TBE on ice. The gel was dried and then exposed on a PhosphorImager and quantified with the Imagequant program.

GST pull-down assay

Each recombinant LTag-GST fusion construct was expressed in the *E. coli* strain XA90. Fifty milliliters of culture yielded ~0.5 mg of protein. After lysis, the cells were spun at 10,000g for 1 h to separate the soluble fraction from the pellet. The supernatant was then incubated with 0.5 mL of glutathione resin for 30 min before being washed with 20 mL of buffer L containing 25 mM Tris (pH 8.0), 250 mM NaCl, and 5 mM DTT to remove contaminating proteins. Approximately 4.0 mg of purified p53 (residues 92–292) was then added to the glutathione column in a volume of 5 mL, and the proteins were incubated for 30 min at 4°C. The column was then washed with 5 mL of buffer L. Twenty microliters of resin from each column was analyzed on a 12% SDS-PAGE gel.

FPLC size-exclusion chromatography

Each recombinant LTag and p53 protein fusion was purified as previously described. The mixture of the two proteins was incubated for 20 min at 25°C. A total of 100 μ L of the protein mixture was then loaded into an analytical grade superdex200 (10/30) column (G.E. Biosciences). The column was run at 0.2 mL per minute in order to improve the accuracy of the size-

exclusion process. The size of the protein complex was estimated using several standard molecular weight markers.

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