Structure of a DNA Polymerase α-Primase Domain That Docks on the SV40 Helicase and Activates the Viral Primosome*§

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DNA polymerase α-primase (pol-prim) plays a central role in DNA replication in higher eukaryotes, initiating synthesis on both leading and lagging strand single-stranded DNA templates. Pol-prim consists of a primase heterodimer that synthesizes RNA primers, a DNA polymerase that extends them, and a fourth subunit, p68 (also termed B-subunit), that is thought to regulate the complex. Although significant knowledge about single-subunit primases of prokaryotes has accumulated, the functions and regulation of pol-prim remain poorly understood.

In the SV40 replication model, the p68 subunit is required for primosome activity and binds directly to the hexameric viral helicase T antigen, suggesting a functional link between T antigen-p68 interaction and primosome activity. To explore this link, we first mapped the interacting regions of the two proteins and discovered a previously unrecognized N-terminal globular domain of p68 (p68N) that physically interacts with the T antigen helicase domain. NMR spectroscopy was used to determine the solution structure of p68N and map its interface with the T antigen helicase domain. Structure-guided mutagenesis of p68 residues in the interface diminished T antigen-p68 interaction, confirming the interaction site. SV40 primosome activity of corresponding pol-prim mutants decreased in proportion to the reduction in p68N-T antigen affinity, confirming that p68-T antigen interaction is vital for primosome function. A model is presented for how this interaction regulates SV40 primosome activity, and the implications of our findings are discussed in regard to the molecular mechanisms of eukaryotic DNA replication initiation.

De novo DNA replication begins with RNA primer synthesis on a DNA template, followed by primer extension and processing DNA synthesis. In prokaryotes, a primosome couples activity of primase with parental DNA unwinding by a hexameric helicase and a single-stranded DNA (ssDNA)β-binding protein, largely through dynamic physical associations among the three proteins (1, 2). In eukaryotes, the core of the primosome is the DNA polymerase α-primase complex (pol-prim), which catalyzes both RNA primer synthesis and extension into RNA-DNA primers (3). Pol-prim initiates synthesis of both leading and lagging strands at eukaryotic replication origins and is required during elongation to initiate synthesis of each Okazaki fragment on the lagging strand. Pol-prim also plays a vital role in telomere maintenance and intra-S phase checkpoint activity. However, the mechanisms that regulate recruitment and activity of pol-prim in these various settings remain poorly understood.

Pol-prim is a complex of four subunits. Its primase subunits (p48 and p58) initially synthesize an 8–12-nucleotide RNA primer, which is then shifted internally to the active site of the associated p180 DNA polymerase subunit for extension into a 30–35-nucleotide RNA-DNA primer (4, 5). The fourth subunit of the pol-prim complex, known as p68 or B-subunit, lacks enzyme activity but is essential for S phase entry in yeast (6) and for p180 accumulation and nuclear import (7–10). The sequence and structure of the C-terminal domain (CTD) of p68 are conserved in the B-subunits of DNA polymerases δ and ε; the p68CTD is tightly associated with the CTD of the p180 subunit (11–13) (see Fig. 1A). In contrast, the N-terminal region of p68 shows little amino acid sequence conservation even with the corresponding pol α-subunits of lower eukaryotes, leading to assumptions that it may be disordered. The N-terminal region of p68 contains a cluster of sites phosphorylated by cyclin-dependent kinase at the G1/S transition (see Fig. 1A) whose precise role in chromosomal replication has not been established (14–16). Indeed, whether the N terminus of p68 is needed for chromosomal replication or cell viability remains questionable (6, 9).

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Pol-prim is essential for replication of SV40 DNA in a complete cell-free reaction reconstituted with purified human proteins (17–19). Detailed analysis of pol-prim in SV40 DNA replication demonstrated that it primes both leading and lagging strand synthesis (20–22). The availability of recombinant human replication proteins and their structures now provides an opportunity to utilize this system to deepen our understanding of the molecular mechanisms underpinning the progression of replication. We have adopted the SV40 model system here to explore the molecular basis for initiation of replication, focusing on the transitions from duplex origin DNA unwinding to priming of the DNA template. As in prokaryotes, transient physical interactions among the helicase, ssDNA-binding protein, and pol-prim enable the SV40 primosome to couple DNA unwinding by the hexameric SV40 T antigen (Tag) helicase to RNA-DNA primer synthesis. Recently, the interacting surfaces of Tag and RPA were mapped in atomic level detail and shown to orchestrate the assembly and disassembly of RPA-ssDNA complexes in subsequent steps of the initiation process (23–25). Moreover, at least three subunits of pol-prim make physical contact with Tag, and these interactions have been implicated in SV40 primosome operation (7, 26, 27). In the presence of ATP or ATPγS, the Tag hexamer interacts with the pol-prim heterotetramer with nanomolar affinity (Kd = ~12 nM) (28, 29). However, definitive evidence for a role of direct pol-prim/Tag contacts in SV40 primosome activity and elucidation of how such contacts might control priming remains elusive.

To develop a better understanding of the role of p68 in the SV40 primosome, we have systematically investigated p68 interaction with Tag using a combination of structural and functional approaches. A 78-residue globular domain at the p68 N terminus (p68N) was identified and shown to be sufficient to bind the Tag helicase AAA+ domain (TagHD). Removal of p68N from the pol-prim complex does not diminish its polymerase or primase activity but nearly abolishes its ability to support initiation of replication from the SV40 origin and to cooperate with Tag to synthesize primers and extend them on RPA-coated template. To gain further insight into p68-Tag interactions, the structure of p68N was determined by solution NMR spectroscopy. A putative Tag-interacting surface of p68N was identified by NMR and confirmed by structure-guided p68N mutations that showed a reduced ability to interact with Tag. The corresponding mutations incorporated into pol-prim resulted in reductions in primosome activity in two different assays. Our data suggest a refined model for SV40 replication initiation, with potential roles for p68N in facilitating pol-prim recruitment, correctly positioning pol-prim to bind and prime the template, and enhancing RNA primer extension into an RNA-DNA primer.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assay—Coding sequences of p68 fragments were amplified by PCR and ligated into the NdeI/BamHI sites of pGBKTK7 vector containing a Leu selection marker. Coding sequences of Tag fragments were amplified by PCR and ligated into the EcoRI/BamHI sites of pGADT7 vector containing a Trp selection marker (Clontech). All of the coding sequences were verified by DNA sequencing. These plasmids were co-transformed into yeast strain AH109, which contains three reporter genes His3, Ade2, and LacZ. The cells were allowed to grow for 4 days on −Leu −Trp plates. Positive colonies were picked and streaked on a −Leu −Trp plate and on a −Leu −Trp −His −Ade plate. The plates were photographed after growth for another 4 days.

Protein Expression and Purification—Pol-prim was expressed in Hi-5 insect cells infected with four recombinant baculoviruses and purified by immunoadfinity chromatography as described previously (30). The pol-prim mutants were purified using the same protocol except that the WT p68 baculovirus was replaced by p68Δ1–78, p68Δ1–107, or I14A baculovi-

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DNA Polymerase Assay—The polymerase activities of the WT and mutant pol-prim were assayed on a randomly primed poly(dA)-oligo(dt) template (20:1) as described previously (30). Specific activity was typically 50 pmol of dTMP/pmol of pol-prim/min.

DNA Primase Assay—The primase activities of the WT and p68 Δ1–107 pol-prim were assayed on single-stranded M13 DNA as described previously (32).

Initiation of SV40 DNA Replication—Monopolymerase assays were carried out as described previously (24) except that pol-prim complexes and amounts varied as indicated in the assays were carried out as described previously (24) except that pol-prim complexes and amounts varied as indicated in the figures and legends.

Primer Synthesis and Elongation in the Presence of RPA—Reaction mixtures (20 μl) containing 100 ng of single-stranded M13 DNA were preincubated with 600–1000 ng of RPA, as stated in the figure legends, in initiation buffer (24) at 4 °C for 20 min. The reactions were then supplemented with 3 μCi of [α-32P]dCTP, 400–600 ng of Tag (or as indicated), and increasing amounts of WT or mutant pol-prim as indicated; incubated at 37 °C for 45 min; and then digested with 0.1 mg of proteinase K/ml in the presence of 1% SDS and 1 mM EDTA at 37 °C for 30 min. Radiolabeled reaction products were then processed and analyzed as described above for the monopolymerase assay.

Isothermal Titration Calorimetry—p68N constructs and Tag 303–627 were buffer exchanged into 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and either 1 mM dithiothreitol (for (H)CC(CO)NH and H(CC)(CO)NH total correlation spectroscopy. 1H,15N distance restraints were generated using two-dimensional homonuclear COSY and (H)CC(CO)NH and H(CC)(CO)NH total correlation spectroscopy. 1H,15N distance restraints were generated using two-dimensional homonuclear COSY, three-dimensional 15N-edited NOESY, three-dimensional 13C,15N-labeled p68N in traditional heteronuclear experiments. Side chain resonance assignments were completed using homonuclear COSY and H(CC)(CO)NH total correlation spectroscopy. 1H,15N distance restraints were generated using two-dimensional homonuclear COSY, three-dimensional 15N-edited NOESY, three-dimensional 13C,15N-labeled p68N in traditional heteronuclear experiments. Side chain resonance assignments were completed using homonuclear COSY and H(CC)(CO)NH total correlation spectroscopy. 1H,15N distance restraints were generated using two-dimensional homonuclear COSY, three-dimensional 15N-edited NOESY, three-dimensional 13C,15N-labeled p68N in traditional heteronuclear experiments. Side chain resonance assignments were completed using homonuclear COSY and H(CC)(CO)NH total correlation spectroscopy. 1H,15N distance restraints were generated using two-dimensional homonuclear COSY, three-dimensional 15N-edited NOESY, three-dimensional 13C,15N-labeled p68N in traditional heteronuclear experiments.
mean square deviation of 2.4 Å (Fig. 2C). Moreover, a Dali search using the Dpoe2NT domain returned the same structures as p68N (37). Despite the similarity in their architecture, amino acid sequence alignment of p68N and Dpoe2NT reveals little sequence similarity, and the chemical nature of their surfaces diverges considerably (Fig. 2D). The p68N structure is characterized by a largely acidic surface with a few small hydrophobic patches, whereas the pol ε domain displays an electrostatic surface with both basic and acidic charge and no significant hydrophobic patches. As will be discussed in more detail below, the surface characteristics of p68N play an important role in mediating interactions with SV40 Tag. The differences in the surfaces of the p68N and Dpoe2NT domains presumably give rise to their functional specificity.

Initiation of SV40 DNA Replication and Primosome Activity Require the p68 N Terminus—We next investigated whether p68N is needed for viral DNA replication. To this end, purified recombinant pol-prim complexes containing a full-length or a truncated p68 subunit were generated and characterized by denaturing gel electrophoresis and Coomassie staining (Fig. 3A). Truncated p68 subunits (lanes 2 and 3) were present in the complex at a stoichiometry equivalent to that of the full-length p68 (lane 1), demonstrating that both truncations retain the ability to stably associate with the p180 polymerase subunit. The polymerase specific activity of the p68(1–107) and WT complexes was identical; the primase specific activity of the p68(1–107) complex was about 2-fold greater than that of the WT (supplemental Fig. S1, A and B). Moreover, truncation of p68 did not compromise the ability of the p180 polymerase subunit. The polymerase specific activity of the Δ1–78 and Δ1–107 (lanes 2 and 3) were present in the complex at a stoichiometry equivalent to that of the full-length p68 (lane 1), demonstrating that both truncations retain the ability to stably associate with the p180 polymerase subunit. 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with unwinding of supercoiled DNA containing the SV40 origin (20). Although accumulation of newly synthesized RNA primers is detectable in this assay in the absence of deoxyribo-nucleotides, primer-dependent extension into RNA-DNA primers in the presence of radiolabeled dCTP amplifies the signal and is hence a more sensitive measure of primer synthesis (20). In the monomerase reaction performed with WT pol-prim, replication products increased in proportion to the amount of pol-prim in the reaction (Fig. 3B, lanes 1–4), and no products were observed in control reactions lacking Tag or pol-prim (lanes 9 and 10). In contrast, little replication was detected with Δ1–107 pol-prim (lanes 5–8). Quantification of the reaction products revealed that the initiation activity of mutant pol-prim was reduced at least 10-fold (Fig. 3B, bottom panel). Similarly, little activity of Δ1–78 pol-prim in initiation of SV40 replication was observed (supplemental Fig. S1D). The results demonstrate a key role for the p68 N terminus in the initiation of replication at the SV40 origin.

In the second assay, natural single-stranded DNA (M13) pre-coated with purified human RPA was used to measure primer synthesis uncoupled from duplex DNA unwinding (24, 38, 39). On this template, purified pol-prim alone does not efficiently generate RNA primers and extend them into radiolabeled RNA-DNA primers. However, the addition of Tag can overcome the inhibition of priming by RPA to permit priming. If p68N-Tag interactions are required for pol-prim activity on RPA-coated template DNA, the activity of Δ1–107 pol-prim should also be compromised in this second assay.

On naked ssDNA template, the ability of mutant and WT pol-prim to synthesize RNA primers and extend them into labeled RNA-DNA primers was comparable (Fig. 3C, lanes 5 and 10), consistent with the similar primase and polymerase enzymatic activity of the two pol-prim complexes. Also as expected, preincubation of the template DNA with saturating amounts of RPA inhibited the activity of all pol-prim complexes (Fig. 3C, lanes 4 and 9). In the presence of Tag, WT pol-prim overcame this RPA inhibition, generating RNA primers and extending them into radiolabeled RNA-DNA products as a function of the amount of pol-prim added (Fig. 3C, lanes 1–3). In contrast, in reactions containing Δ1–107 pol-prim and Tag, radiolabeled products did not increase beyond the level observed in reactions without Tag (Fig. 3C, lanes 6–8). Quantification of the reaction products confirmed that truncation of the p68 N terminus nearly abolished primosome activity (Fig. 3C, bottom panel). The results indicate that the N-terminal region of p68 is vital for SV40 primosome activity.

The Tag-interacting Surface of p68N—To establish a means to determine the functional role of p68N in the SV40 primosome, we set out to measure the affinity of p68N interaction with Tag and map the p68N surface that interacts with Tag. The technique of isothermal titration calorimetry was used to obtain accurate binding parameters. Fitting of the binding isotherm for the titration of p68N into a solution of a monomeric Tag 303–627 construct (31) provided a dissociation constant of 0.1 μM (Fig. 4A). An affinity in the micromolar range is consistent with the proposed role for p68N in mediating the interaction between pol-prim and Tag as part of the dynamic assembly/disassembly of proteins in the primosome.

NMR spectroscopy was then applied to identify p68N residues in the binding interface with Tag 303–627. Titration of a stoichiometric amount of unlabeled Tag 303–627 into a solution of 15N-enriched p68N provided a 15N-1H HSQC spectrum in which the resonances were severely broadened. The loss of signals precludes the use of the powerful NMR chemical shift perturbation approach for analysis of binding surface. However, in these situations an alternative approach can be applied based on detection of differential reduction in signal intensity for residues in the binding interface at substoichiometric ratios (40). Fig. 4B shows the normalized intensities of the NMR signals for the Tag 303–627:p68N complex at a ratio of 0.1:1. TABLE 1

| TABLE 1 Structural statistics of p68N |
|---------------------------------------|
| Total restraints | 1144 |
| NOE restraints | 1046 |
| Short range | 593 |
| Medium range | 221 |
| Long range | 230 |
| Dihedral angle restraints | 100 |
| Restrainment violations (means ± S.D.) | |
| Distance restraints 0.2 Å | 0 |
| Dihedral angle constraint violations (≥5°) | 0 |
| Maximum distance restraint violation | 0.08 ± 0.01 |
| Maximum dihedral restraint violation | 0.1 ± 0.4 |
| AMBER energies (kcal/mol) | |
| Restrainment energy | 1.1 ± 0.2 |
| Total energy | −2911 ± 12 |
| Ramachandran statistics (%) | |
| Most favored | 92.0 |
| Additionally allowed | 6.3 |
| Generously allowed | 0.8 |
| Disallowed | 0.9 |
| Root mean square deviation from mean structure (Å) | |
| Backbone atoms | 0.60 ± 0.12 |
| Heavy atoms | 1.00 ± 0.10 |

* Residues used for root mean square deviation calculations include Ala¹–Glu⁹².
When the residues whose intensities are most significantly reduced are mapped onto the p68N structure, they identify a putative Tag-binding surface (Fig. 4C). This surface maps to a hydrophobic patch surrounded by negative charge. The residues in this putative Tag-binding surface are ideal candidates for site-directed mutagenesis experiments to probe the functional role of p68N in the primosome. Toward this end, a series of site-directed substitution mutations in p68N was designed, and yeast two-hybrid assays were performed to screen for their effects on Tag binding. The WT p68N interacted effectively with TagHD, as evidenced by growth in the absence of histidine and adenine, whereas no growth was observed with the empty vector (sectors 1–3) (Fig. 5A). In contrast, substitution of p68N Ile14 by Ala or Glu (sector 4, not shown) or of Phe15 by Arg (not shown) abolished interaction with Tag. Interestingly, the F15A mutation reduced binding to TagHD under physiological conditions, whereas the F15Y substitution still interacted with TagHD (sectors 6, 7). Additional amino acid substitutions at Glu11, Glu39, or Glu44 of p68N abolished interaction with TagHD, whereas substitutions at several other residues in p68N had little effect (Fig. 5B). Pull-down assays performed at low ionic strength confirmed that Ile14 substitution by Ala or Val and Phe15 substitution by Ala or Tyr weakened the interaction of p68N(1–107) with TagHD (Fig. 5C, lanes 3–6). The p68N I14A substitution displayed a similarly reduced interaction with TagHD, compared with that of WT p68N, at KCl concentrations ranging from 10 to 150 mM.6

A reduction in mutant p68N interaction with TagHD can arise from the desired perturbation of the interaction surface with TagHD or through perturbation of the structure or loss of structural stability. Therefore, before proceeding to functional analysis, it was imperative to confirm that the effects of amino acid substitutions on the structure of p68N are minimal. To this end, we prepared four 15N-enriched mutant proteins (E11K, E39K, E44K, and I14A) and assayed their structures by 15N-1H HSQC NMR. Indeed, comparisons with the WT p68N revealed that the three Glu to Lys substitutions led to small but widespread effects on the NMR spectrum, indicating possible structural perturbations (not shown). However, the I14A substitution had much smaller effects on the p68N spectrum (Fig. 5D), providing confidence that its structure was not significantly perturbed. To verify the success of the mutant design, the effect of the I14A mutation on Tag interaction was investigated by isothermal titration calorimetry. In contrast to the WT protein, p68N(I14A) showed no detectable heat of

6 H. Huang, unpublished observations.
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![Diagram](image)

**FIGURE 4.** p68N interacts with Tag 303–627. A, isothermal titration calorimetry titration of p68N into Tag 303–627. The data were fit to a single-site binding model, which provided a 

The Interacting Domains of p68 and Tag—If p68-Tag interaction is vital for SV40 DNA replication, the weakened interaction of I14A p68 with Tag would be expected to diminish replication but not abolish it. To test this prediction, a pol-prim with the I14A substitution in p68 was constructed, purified, and characterized (Fig. 6A). As expected, the substitution had little effect on the specific activity of pol-prim in polymerase or primase enzyme assays (not shown). The single-residue substitution had little effect on the specific activity of pol-prim in polymerase or primase enzyme assays (not shown).

The Interacting Domains of p68 and Tag—If p68-Tag interaction is vital for SV40 DNA replication, the weakened interaction of I14A p68 with Tag would be expected to diminish replication but not abolish it. To test this prediction, a pol-prim with the I14A substitution in p68 was constructed, purified, and characterized (Fig. 6A). As expected, the substitution had little effect on the specific activity of pol-prim in polymerase or primase enzyme assays (not shown).

**DISCUSSION**

The Interacting Domains of p68 and Tag—Progress in understanding the initiation of eukaryotic replication is currently limited by the lack of knowledge of the structural basis for the recruitment of pol-prim and delivery of the template strand to it for priming. Here we identified a previously unrecognized N-terminal globular domain in the p68 subunit of pol-prim, of the nature observed here for p68N-TagHD.

The structure of the p68N domain is closely related to that of the NTD of pol ε Dpo2 and subdomains of other AAA + proteins (Fig. 2C), reflecting an evolutionary relationship (37). AAA + proteins function in multiple steps of DNA replication as origin-binding proteins, helicases, helicase loaders, and clamp loaders (43). Interestingly, the AAA + protein DnaC from *E. coli* is needed in conjunction with the hexameric DnaB helicase to enable primer synthesis by DnaG on ssDNA template coated by the *E. coli* SSB protein but not on naked ssDNA template (44). Thus, docking of p68N with Tag in the SV40 primosome (Figs. 3 and 6) may play a role analogous to that of DnaC in the prokaryotic primosome (44).

The data presented here are derived from a viral model system, but it seems unlikely that the p68N domain could have been evolutionarily selected solely to enable viral primosome activity. A more likely possibility is that p68N may interact with cellular proteins to enable primer synthesis in chromosomal replication, but to our knowledge this has not been reported. The absence of significant amino acid sequence homology between p68N and Dpo2 NTD and the distinctly different chemical nature of their surfaces suggest that they have diverged and likely interact with different cellular partner proteins. Powerful precedents for structural and functional conservation of DNA processing proteins without significant amino acid sequence homology are well known, e.g. proliferating cell
nuclear antigen and β-clamp (45). The structural similarity of p68N and Dpo2 NTD suggests that Dpo2 NTD also serves as a docking module, directly or indirectly facilitating interaction of polymerase with the Mcm2–7 complex at replication origins.

Model for Recruitment of Pol-prim to Initiate Priming on RPA-coated Template DNA—In previous studies, we obtained evidence using reconstituted SV40 replication reactions that the hexameric Tag helicase interacts with two molecular surfaces of RPA (23–25). A basic surface of the origin-binding domain (OBD) (residues 131–259) of Tag binds to an acidic surface of RPA70AB; as ssDNA is extruded from the helicase, a ternary complex of OBD-70AB-ssDNA assembles transiently. Upon extrusion of additional ssDNA, formation of the RPA-ssDNA complex leads to release of Tag-ssDNA for additional RPA loading, thus coupling origin unwinding to RPA-ssDNA filament formation (23). Conversely, to facilitate priming of RPA-bound template by pol-prim, transient physical interactions between the basic surface of the OBD of one subunit of a Tag hexamer with RPA32C and the same surface of a second Tag subunit with an acidic surface of RPA70AB lead to remodeling of the ssDNA binding mode of RPA (23–25) (Fig. 7, top panel). The remodeled ssDNA binding mode, in which RPA occludes only 8–10 nucleotides, transiently exposes ssDNA template that can be utilized for primer synthesis (Fig. 7, middle panel).

This model can be extended based on the data described here, which show that physical interactions of pol-prim with TagHD enable primase recruitment to the exposed ssDNA. Each hexamer of Tag helicase associates with one pol-prim complex (28). Previous work demonstrated the interaction of Tag with p180 and p68 and suggested their involvement in the initiation of replication (7, 27). Our combined structural and functional analysis of these interactions now reveals that the N-terminal domain of p68 (residues 1–78, p68N) docks on the surface of TagHD and is tethered through a long, presumably disordered linker to the conserved CTD of p68 (residues 206–598) (Fig. 1A). The C-terminal domain of p68, in turn, associates tightly but flexibly with the C-terminal zinc domain of p180 (13). The zinc domain of p180 also binds to the primase heterodimer (4).

The data in Figs. 3 and 6 imply that p68N interaction with TagHD is crucial for pol-prim recruitment to the exposed ssDNA. Each hexamer of Tag helicase associates with one pol-prim complex (28). Previous work demonstrated the interaction of Tag with p180 and p68 and suggested their involvement in the initiation of replication (7, 27). Our combined structural and functional analysis of these interactions now reveals that the N-terminal domain of p68 (residues 1–78, p68N) docks on the surface of TagHD and is tethered through a long, presumably disordered linker to the conserved CTD of p68 (residues 206–598) (Fig. 1A). The C-terminal domain of p68, in turn, associates tightly but flexibly with the C-terminal zinc domain of p180 (13). The zinc domain of p180 also binds to the primase heterodimer (4).

The data in Figs. 3 and 6 imply that p68N interaction with TagHD is crucial for pol-prim recruitment to RPA-ssDNA and primer synthesis. Several plausible roles for Tag-p68N interaction in primosome activity can be envisioned. In the simplest model, the weak interactions of both p68N and p180N with TagHD may be needed to form a sufficiently high affinity interaction to allow pol-prim to bind to the naked ssDNA exposed by RPA binding to Tag OBD. In the absence of p68N, the remaining interaction of TagHD with p180 may be too weak and transient to support primosome activity. Although qualitative in nature, pull-down reactions conducted under conditions

FIGURE 5. Structure-guided mutational analysis of the Tag-binding surface of p68N. A and B, single residue substitutions in p68(1–107) were screened in yeast two-hybrid assays for interaction with GST-TagHD. Left panel, control plate; right panel, growth on the selective plate indicates interaction. The numbered sectors are identified in the tables below. C, glutathione (G) beads were incubated with 10 μg of WT His-tagged p68(1–107) or mutants I14A, I14V, F15A, and F15Y as indicated in the presence (+) or absence (−) of GST-TagHD (lanes 1–6). Proteins bound to the beads were detected by Western blotting with anti-His or anti-GST antibody. Lane 7, input (IN) p68(1–107) (200 ng). D, the p68N II4A domain maintains the WT fold. 15N-1H HSQC spectra were collected for WT (red) and I14A (blue) p68N. The spectra overlay very well, indicating that no major structural perturbation was induced by the I14A mutation.
similar to those in the cell-free replication assay suggest that Tag interaction with Δ1–107 and Δ1–78 pol-prim is similar to that with WT pol-prim (supplemental Fig. S1C). These observations suggest that binding of p180 and primase to Tag are independent of p68N and, in combination, are of sufficient affinity to generate a stable complex (28). Nonetheless, the decrease in primosome activity of p68 mutant pol-prim in two different assays directly correlates with the decrease in p68N-Tag interaction affinity (compare Figs. 1 and 3 with Figs. 5 and 6). Taken together, these results argue that the role of p68N docking with TagHD in primosome activity is important but probably not solely to increase the affinity of interaction.

A second potential role for independent docking sites for p68N, p180, and primase on Tag may be to position the primosome subunits spatially to access the template DNA exposed through Tag-directed remodeling of the RPA ssDNA binding mode (Fig. 7, middle and lower panels). In this scenario, loss of the p68N contact with TagHD may result in an overly mobile TagHD/pol-prim assembly that is unable to position primase properly to bind to the exposed template or to maintain it in that position long enough to form a primer. Consistent with this possibility, the p68CTD-p180CTD interaction module observed in cryo-electron microscopy (13) displays considerable flexibility that might be constrained by docking the N termini of the two subunits at separate sites on Tag-HD. Similarly, the length of the tethers that link the p68CTD-p180CTD module with p68N and p180 residues 189–313, which dock on Tag (27), may be important to position primase on the template. Docking of p68N on TagHD may also be important for temporal coordination of priming with origin DNA unwinding by Tag, as well as strand separation during fork progression. Initial indications of temporally coordinated helicase-primase movement came from the observation that the rate of DNA unwinding by Tag helicase is slowed in the presence of pol-prim (29, 46). This inhibition was observed using supercoiled SV40 origin DNA, as well as on model fork templates, and depended on pol-prim concentration. Notably, pol-prim inhibition of Tag helicase was shown to require the p48-p58 complex, the p180-p68 complex, and human RPA, mirroring the requirements for SV40 primosome function, but did not require oligoribonucleotide synthesis (46). These findings suggest that physical interaction of pol-prim with Tag helicase may cause its translocation, possibly to allow for priming, as reported for prokaryotic helicase-primase assemblies (1, 47, 48).
Mcm2–7 hexamers on duplex DNA (52, 53) is strikingly similar to the assembly of Tag helicase on the SV40 origin (54, 55). Yet how the Mcm2–7 complex, upon activation of DNA unwinding, associates with DNA polymerase α-primase for primosome activity remains poorly understood.

The crucial role for human p68 in the initiation of SV40 DNA replication would be consistent with the requirement for the corresponding pol α-subunit B (Pol12) for the G1/S transition and cell viability in budding yeast (6). Interestingly, although the N terminus of yeast Pol12 shows little amino acid sequence conservation with human p68N, computational prediction of its structure reveals a similar four-helix bundle (data not shown). However, in-frame deletions within the predicted N-terminal domain of the yeast B-subunit did not reduce cell viability or growth rate (6), raising the question of whether the Pol12 N terminus contributes to initiation of replication in yeast. It remains possible that the Pol12 N terminus docks with an interaction partner in the replisome but that loss of the interaction might not lead to an obvious phenotype in a wild type genetic background. This would be reminiscent of yeast strains lacking Ctf4, which are viable (56, 57), even though binding of And-1 (known as Ctf4 or Mci1 in yeast) to p180 is now known to serve a vital role in pol-prim recruitment and in replisome progression in organisms from yeast to human (58–64). Further analysis is needed to explore a potential role for p68N in initiation of chromosomal replication under more stringent conditions. In addition, primosome activity at telomeres (65, 66) and at sites of stalled replication forks (67, 68) is vital for genomic stability but remains poorly understood. Our work suggests that a role for p68N should be investigated.

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Implications for Pol-prim Function in Chromosomal DNA Processing—The essential helicase in eukaryotic chromosomal replication forks is the Mcm2–7 hexamer in complex with Cdc45 and GINS proteins (49–51). Like Tag, the Mcm2–7 helicase assembly unwinds duplex DNA as it translocates 3’→5’ on the leading strand template, displacing the lagging strand template. Moreover, the concerted head-to-head assembly of two
