# INSIGHTS INTO THE ROLES OF HUMAN CDC6 AND REPLICATION PROTEIN A IN INITIATION OF EUKARYOTIC DNA REPLICATION

By

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"Praise be to the name of God for ever and ever…"

Daniel 2:20

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| | |
|---|---|
| A | Adenosine or Alanine |
| aa | Amino acid |
| ADP | Adenosine 5'-diphosphate |
| ATP | Adenosine 5'-triphosphate |
| ATPγS | Adenosine 5'-γ-thiotriphosphate |
| bp | Base pairs |
| BrdU | Bromodeoxyuridine |
| BSA | Bovine serum albumin |
| C | Cytidine/cytosine |
| °C | Degrees Celsius |
| Ci | Curie |
| Cdc | Cell division cycle |
| Cdk | Cyclin-dependent kinase |
| cDNA | Complementary DNA |
| CNBr | Cyanogenbromide |
| $CO_2$ | Carbon dioxide |
| cpm | Counts per minute |
| CTD | Carboxyl-terminal domain |
| CTP | Cytidine 5'-triphosphate |
| Da | Dalton |
| dATP | Deoxyadenosine 5'-triphosphate |

| | |
|---|---|
| DBD | DNA binding domain |
| dCTP | Deoxycytidine 5'-triphosphate |
| dGTP | Deoxyguanosine 5'-triphosphate |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic Acid |
| dNTPs | Deoxynucleoside triphosphate |
| dsDNA | Double-stranded DNA |
| DTT | Dithiothreitol |
| dTTP | Deoxythymidine 5'-triphosphate |
| $dT_{30}$ | Oligodeoxythymidylate |
| E | Glutamic acid |
| E. Coli | Escherichia coli |
| EDTA | (Ethylenedinitrilo)tetraacetic acid |
| EGTA | Ethylene glykol-bis-(□-aminoethylether)-N,N'-tetraacetic acid |
| EM | Electron microscopy |
| FBS | Fetal Bovine Serum |
| FCS | Fetal Calf Serum |
| G | Guanidine/guanosine |
| g | Gram or gravitational constant |
| gp | Gene protein |
| GST | Glutathione S-transferase |
| GTP | Guanosine 5'-triphosphate |

| | |
|---|---|
| h | Hour |
| h | Human |
| HCl | Hydrochloric acid |
| HEPES | N-[2-hydroxyethyl] piperazine-N'-[2ethansulfonic acid] |
| His | Histidine-tagged |
| IgG | Immunoglobulin M |
| IPTG | Isopropyl □-D-thiogalactopyranoside |
| K | Lysine |
| KCl | Potassium chloride |
| kDa | Kilodalton |
| Kpi | Potassium phosphate |
| KOH | Potassium Hydroxide |
| L/l | Liter |
| LB | Luria broth |
| LiCl | Lithium chloride |
| M | Molar (mol/L) |
| mAmps | Milliamperes |
| MCM | Minichromosome maintenance |
| mg | Milligram |
| Mg | Magnesium |
| $MgCl_2$ | Magnesium Chloride |
| min | Minute |
| ml | Milliliter |

| | |
|---|---|
| mM | Millimolar |
| mRNA | Messenger ribonucleic acid |
| □Ci | Microcurrie |
| □g | Microgram |
| □l | Microliter |
| □M | Micromolar |
| NaCl | Sodium Chloride |
| NES | Nuclear Export Signal |
| NLS | Nuclear Localization Signal |
| Ni-NTA | Nickel-nitrilotriacetic acid agarose |
| ng | Nanogram |
| NLS | Nuclear Localization Signal |
| nm | Nanometer |
| nmol | Nanomol |
| NP-40 | Nonidet-P 40 |
| nt | Nucleotide |
| OBD | Origin Binding Domain |
| OB fold | Oligosaccharide/oligonucleotide binding fold |
| OD | Optical density |
| ORC | Origin Recognition Complex |
| ON | Overnight |
| P | Proline |
| P-loop | Phosphate-binding loop |

| | |
|---|---|
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate Buffered Saline |
| pBS | pBluescript |
| PCNA | Proliferating Cell Nuclear Antigen |
| PCR | Polymerase Chain Reaction |
| PIPES | Piperazine-N,N'-bis-2-ethansulfonyl acid |
| Pfu | Plaque forming unit |
| pmol | Picomol |
| PMSF | Phenylmethylsulfonyl fluoride |
| Pol $\alpha$ | Polymerase $\alpha$ |
| Pol $\delta$ | Polymerase $\delta$ |
| Pol $\varepsilon$ | Polymerase $\varepsilon$ |
| pol-prim | DNA polymerase $\alpha$-primase |
| pre-RC | Pre-Replicative Complex |
| Q | Glutamine |
| R | Arginine |
| RC | Replicative Complex |
| RFC | Replication Factor C |
| RNA | Ribonucleic acid |
| RPA | Replication protein A |
| rpm | Revolutions per minute |
| RT | Room temperature |
| S | Serine |

| | |
|---|---|
| Sc | Saccharomyces cerevisiae |
| SDS | Sodium dodecyl sulfate |
| sec | Second |
| Sp | Saccharomyces pombe |
| SSB | Single-strand binding protein |
| ssDNA | Single-stranded DNA |
| SV40 | Simian Virus 40 |
| T | Thymidine or Threonine |
| Tag | T antigen |
| TBE | Tris borate EDTA buffer |
| TBP | TATA-binding protein |
| TBS | Tris buffered saline |
| TBST | Tris buffered saline tween-20 |
| TEMED | N,N,N',N'-Tetramethylethylenediamin |
| TCA | Trichloroacetic Acid |
| Tris | Tris-(hydroxymethyl-)aminomethane |
| U | Unit |
| UTP | Uridine 5'-triphosphate |
| V | Volt |
| wt /WT | Wild type |

CHAPTER I

# DECONSTRUCTING THE REPLISOME

## Introduction

Since the discovery of the structure of DNA as an information-bearing molecule of the cell in 1953 by Watson and Crick, considerable effort was directed towards elucidating the mechanism by which DNA replication occurs. A simple and elegant model immediately suggested a mechanism for DNA duplex replication. The seemingly simple and straightforward mechanism for initiation of DNA replication proved to be complicated by the number of factors that comprised a finely tuned replication machinery whose elements engage in elaborate interactions and replace each other on a DNA molecule for a successful and error-proof initiation of replication to ensue. Ten years after the DNA structure was elucidated, Jacob, Brenner, and Cuzin proposed the "replicon" hypothesis in which a defined unit of DNA (replicator) was envisioned carrying out independent replication (Jacob et al. 1963). The replicator, in turn, is recognized by an "initiator"—a diffusible protein able to recognize the replicator (Figure 1). This model predicted the existence of origins of replication proximal to the replicator, where initiation of replication would begin, and an initiator protein that is

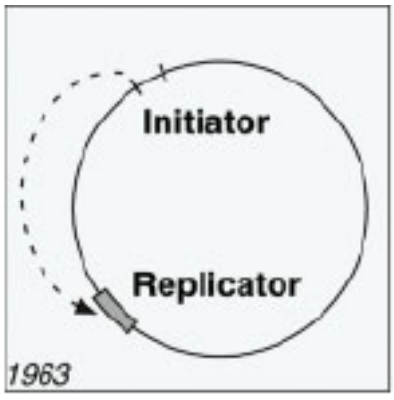


**Figure 1. Replicon model as originally proposed by Jacob, Brenner and Cuzin in 1963**

able to open the DNA duplex. Indeed, the proposed model proved to be correct. Both replicators and initiators were subsequently identified in all biological kingdoms: from viruses and bacteria to humans. Many viral and bacterial origins were shown to be simple well-defined single origin systems (Gilbert 2004). In higher-order organisms, however, the situation has proven to be much more complex. Budding yeast origins termed autonomously replicating sequences (ARSs) are fairly short, less than 200 bp, and contain essential elements that contribute to their replication activity. Fission yeast origins are much larger and demonstrate much more complex structure, covering around 750 bp and containing more elusive and less experimentally defined elements. This trend continues as the complexity of an organism increases. Some human origins (e.g. human β-globin or hamster DHFR locus) stretch over tens of thousands of base-pairs and contain multiple genetic and epigenetic elements, whose functions often remain elusive (Mendez and Stillman 2003).

Despite the great diversity of replicators, initiators are conserved throughout the phylogenetic tree, if not in identity, at least in function. Several initiator activities can be defined: origin recognition, duplex melting, DNA unwinding, ssDNA protection, torsional stress relief, RNA priming, and nascent strand elongation. A function in many cases does not correspond to a single protein but rather a complex of proteins that interact in a defined order and manner. Most homologues of proteins that carry out these activities in model organisms have been identified today. They have been collectively called a replisome (Baker and Bell 1998). The current model of eukaryotic replication based on genetic studies in yeast, biochemical studies in *Drosophila*, *Xenopus,* and several *in vitro* replication assays define the steps necessary for initiation of replication to

ensue: pre-RC formation and its activation to pre-IC (Dutta and Bell 1997; Waga and Stillman 1998; Bell and Dutta 2002; Nishitani and Lygerou 2002; Machida and Dutta 2005). Pre-replication complexes are formed in late M or early $G_1$ phase of the cell cycle when CDKs are inactive (Figure 2). Pre-RC formation, also termed origin licensing, begins when a complex of six origin recognition proteins (ORCs) binds to origins of replication and melts the DNA.

ORC then recruits Cdc6, an AAA+ family protein that is a putative helicase loader. Multi-species sequence alignment of Cdc6, Orc1, and replication factor (RFC), a known replication clamp loader, revealed considerable similarity over a wide region, which helped to elucidate Cdc6 function in replication (Perkins and Diffley 1998). The structure of archaeal Cdc6 was solved by x-ray crystallography and the results corroborated homology of Cdc6 protein with clamp loader proteins (Liu et al. 2000). With assistance from another initiation factor (Cdt1), Cdc6 is required for loading of the MCM2-7 complex of proteins that forms a double hexamer on DNA. MCM2-7 has been considered for a number of years as a putative helicase, but major helicase activity was absent from the complex and only non-processive helicase activity was observed from a MCM 4,6,7 double trimer. Recent studies provide strong evidence in support of a new candidate for eukaryotic replicative helicase. A gene MCM8, not related to MCM2-7, was identified in human cells that exhibits cellular activity reflective of replicative helicase: its gene product is bound to chromatin in S phase of the cell cycle, it possesses robust helicase activity *in vitro*, and it moves with replication forks (Gozuacik et al. 2003). This year's investigation of a *Xenopus* homolog of human Mcm8 protein

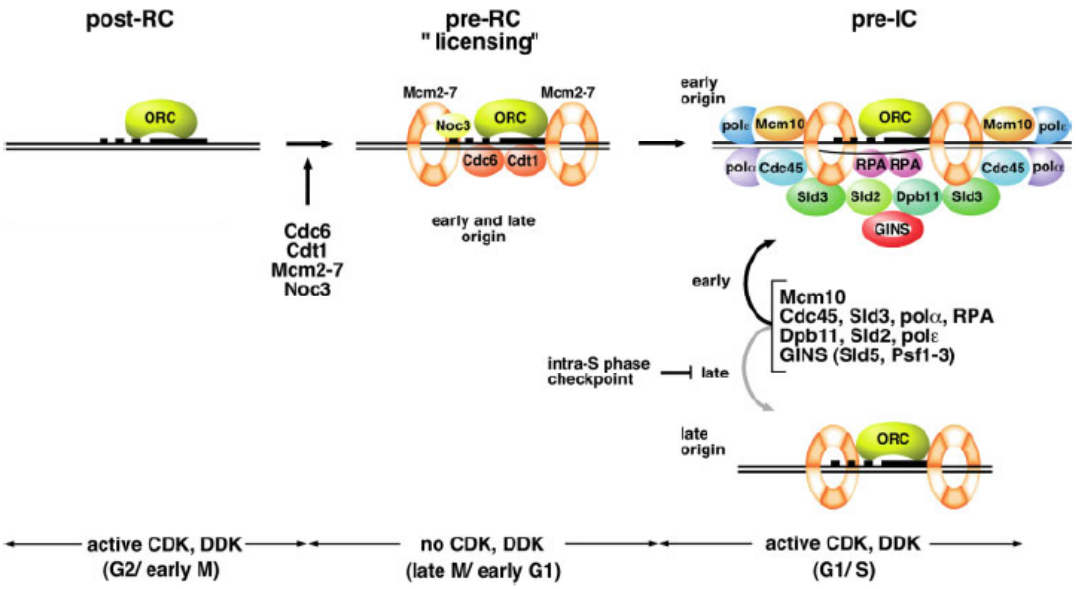


**Figure 2. Two-step activation of a eukaryotic origin of replication.** Multiple initiator proteins assemble at the origins of replication during the cell cycle. In the post-RC (non-competent) state, ORC is bound to the replicator sequences (darker boxes represent the conserved A, B1, B2, B3 elements identified in well-characterized yeast origins of replication; (Laskowski et al. 1996; Kelly and Brown 2000)). Origin licensing occurs during a window of the cell cycle with little or no CDK activity when Cdc6, Cdt1, Noc3 and MCM proteins are incorporated and form a pre-RC structure. Upon activation of CDKs and DDK, the pre-RC is changed to a pre-IC by the removal of Cdc6 and Cdt1 and the successive incorporation of multiple initiators. The architecture of the pre-IC shown is tentative. Formation of pre-ICs at the different origins follows a temporal program. Pre-IC formation at late origins is prevented by the action of intra-S phase checkpoint mechanisms. Adapted from Mendez and Stillman, 2003.

confirmed previous results and provided further evidence for Mcm8's function in the elongation step of replication (Maiorano et al. 2005). Two other proteins, Noc3 and Yph1, were shown to be essential for S-phase progression, the ability of Cdc6 to load MCM2-7, and pre-RNA processing, but their biochemical function in DNA replication is still elusive (Milkereit et al. 2001; Du and Stillman 2002; Zhang et al. 2002). Loading of MCM2-7 complex at the origin marks the formation of pre-RC and licensing of the origin for replication.

Even though origins are licensed at the $G_1$/S transition, action of a number of kinases including cyclin-dependent kinases (CDKs), Dbf4-dependent kinases (DDK), Rad 53, Mec1, and the assembly of the second wave of initiation factors are required for replication to begin. This step is termed pre-initiation complex (pre-IC) assembly and is defined by dissociation of Cdc6 and Cdt1 from the chromatin (Bell and Dutta 2002; Tanaka and Diffley 2002). A number of factors are required for the loading of polymerases after an origin is activated. Among them are Mcm10 and Cdc45. Mcm10 is an abundant protein that acts after MCM2-7 complex is loaded at the pre-IC and is essential for Cdc45 and RPA recruitment in *Xenopus* system. Initial studies suggested that *S. cerevisiae* Mcm10 is required for MCM2-7 recruitment to chromatin during $G_1$, suggesting an earlier step in Mcm10 function (Homesley et al. 2000). However, later studies in budding, fission yeast, *Xenopus*, and human suggested a later step after pre-RC formation and was shown to be required for Cdc45 association with chromatin and polymerase stabilization (Wohlschlegel et al. 2002; Gregan et al. 2003; Izumi et al. 2004; Ricke and Bielinsky 2004; Sawyer et al. 2004). Cdc45 was found to associate with the origins upon the activation of CDK and DDK, it directly interacts with pre-IC

components, and it is required for the elongation of nascent DNA (Zou and Stillman 1998; Aparicio et al. 1999; Tercero et al. 2000; Zou and Stillman 2000). At a later step, another group of proteins interacts with the origins to promote the replicative activity of polymerases. This group includes the Dpb1, Sld, Psf, and GINS proteins that were shown to interact with polymerase ε, Cdc45, and to be required for pol-prim association with chromatin (Araki et al. 1995; Masumoto et al. 2000; Kamimura et al. 2001; Takayama et al. 2003).

The presence of RPA on ssDNA is required for the further steps of cellular DNA replication. Pol-prim complex that lacks processivity must be replaced by processive polymerase δ (pol δ). Polymerase switching occurs during the synthesis of both leading (Tsurimoto et al. 1990) and lagging strands (Waga and Stillman 1994). (Figure 3).

A short (~30 nt) chimeric RNA/DNA strand generated by pol-prim is termed initiator DNA (iDNA) (Eliasson and Reichard 1978; Bullock et al. 1991). RFC recognizes the 3' end of iDNA and displaces pol-prim. Then, RFC triggers the formation of the primer recognition complex, which includes proliferating cell nuclear antigen (PCNA) recruitment followed by pol δ. PCNA, a ring-shaped tether, increases processivity of pol δ to 5-10 kb or until it encounters a previously synthesized Okazaki fragment (Matsumoto et al. 1990; Tsurimoto et al. 1990; Eki et al. 1992).RNA primers are then excised by RNase HI and FEN1 nucleases, the gap is filled by pol δ, and the nick closed by DNA ligase I (Waga and Stillman 1998). (Figure 3).

Increased complexity of replication mechanism in the higher order eukaryotes allows for a finer regulation potential of DNA replication and multiple failsafe mechanisms *in vivo*. At the same time, the complexity delayed the use of higher

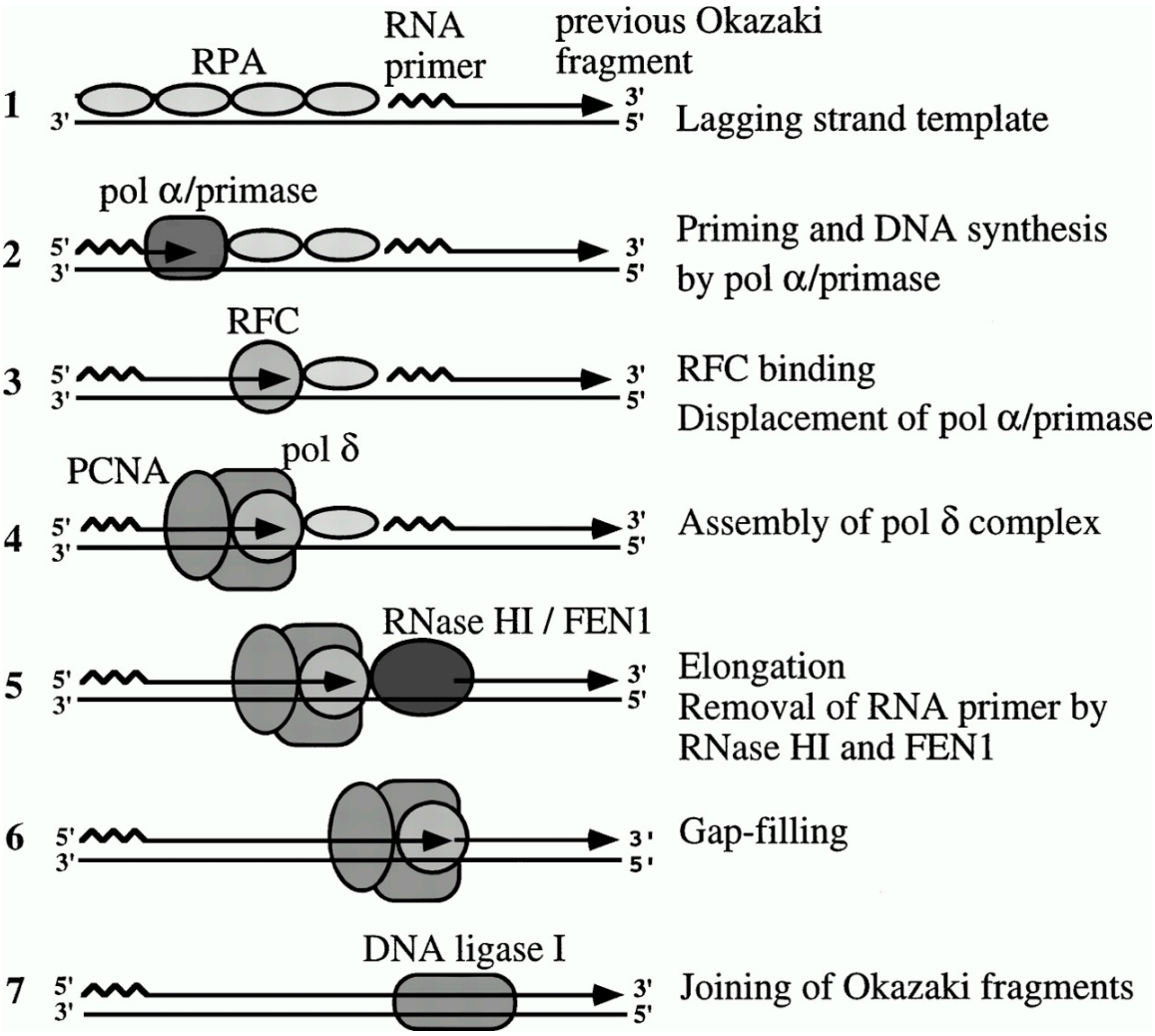


**Figure 3. Polymerase switching and maturation of Okazaki fragments on a lagging-strand DNA template.** Adapted from Waga and Stillman, 1998.

eukaryotes as a model organism for the study of DNA replication (Laskey et al. 1977; Coleman et al. 1996; Walter et al. 1998; Chesnokov et al. 1999). Therefore, the development of a simple effective *in vitro* replication system was essential to understanding the basic mechanism of replisome function (Challberg and Kelly 1989; Stillman 1989; Waga and Stillman 1994). One of the best-studied systems used to investigate initiation of DNA replication is Simian Virus 40 (SV40) *in vitro* assay.

**Modus operandi of simian virus 40**

The limitations of replication systems of higher eukaryotes can be overcome by the use of a viral based replication system, in which one or several viral proteins are able to recruit cellular replication components and initiate replication on the viral origin, which is simple and well defined. A much greater level of understanding of the replication factors and mechanisms is usually achieved for such a system. A wide range of assays, reagents, and sometimes even structural data are available for a viral *in vitro* system. One of the best-characterized and studied replication systems is simian virus 40 (SV40) (Fanning and Knippers 1992; Herendeen and Kelly 1996; Bullock 1997; Stenlund 2003; Simmons et al. 2004).

SV40 is indigenous to Asian macaques and infects several species asymptomatically, but is able to transform rodents and human cell culture. SV40 is a double-stranded circular DNA virus of approximately 5 kb that is assembled into a mini-chromosome using cellular histones and possesses a well-defined single origin of replication. The viral origin is a part of a control region that spans approximately 300 bp and contains early and late transcriptional regulatory regions (Figure 4). The genes

encoding the large T-antigen (Tag) and the small T-antigen are regulated by the early promoter, while genes encoding structural proteins VP1, VP2, and VP3 are controlled by the late one. Due to the early regulatory region's similarities to human promoters, they do not require viral proteins to initiate transcription. Large Tag contains a Nuclear Localization Signal (NLS) and thus accumulates in the nucleus upon expression. There it activates the late promoter and inhibits its own production through a feedback loop. One of the major roles of Tag is to inhibit functions of the p53 and pRB family of tumor suppressor proteins, thereby facilitating cell cycle entry, development of cellular chromosomal aberrations, and genome instability. Small Tag activates cell proliferation by binding to catalytic and regulatory subunits of protein phosphatase PP2A, which in turn activates the β-catenin pathway and allows infectivity. Since small Tag is not involved in SV40 DNA replication *in vitro*, it is of limited interest for study of DNA replication in higher eukaryotes and will not be described further.

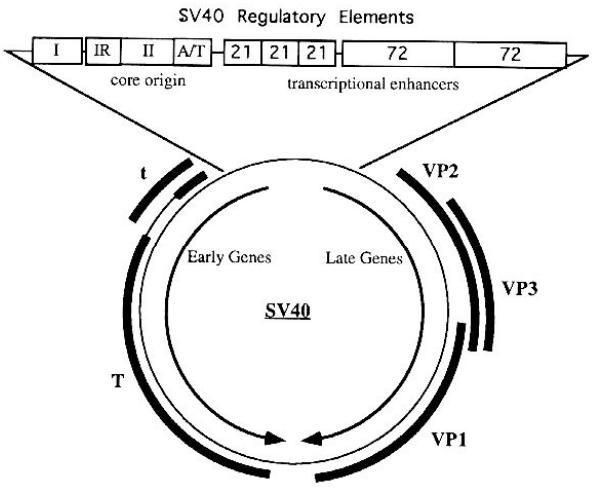


**Figure 4. Diagram of the circular SV40 genome.** Early and late mRNAs are depicted as arrows pointing in the 5' to 3' direction. The regions encoding the early and late gene products are depicted as thick arcs and labeled accordingly. The intron of the large T antigen is shown as a thin arc connecting the T-encoding regions. The SV40 regulatory element controlling transcription and replication is magnified above. The figure and the legend are adapted from Herendeen and Kelly, 1996.

**The mechanism of viral DNA replication**

The seemingly simple model of SV40 replication still presents a challenge to a careful investigator who would like to know the inner workings of the viral replication machinery. Although the protein components of the replisome are known and the general order of their assembly on the viral origin has been determined, the nature of protein interactions and molecular mechanisms of these proteins remain elusive. The commonly accepted model of the viral initiation of replication is as follows. Upon entry into a cell, the SV40 viral genome is released from the capsid as a mini-chromosome containing cellular histones that make it indistinguishable from cellular chromosomes (for a review see (Herendeen and Kelly 1996; Bullock 1997)). The early viral promoter is self-sufficient and does not require viral gene products to initiate replication. Large Tag mRNA is one of the first to be transcribed by the early promoter and the protein orchestrates the progression of further events in the viral infection. Having a NLS, Tag immediately localizes to the nucleus where it induces expression of a set of host genes to drive the cell into S phase where viral replication is the most efficient. At the same time, Tag sequesters cellular DNA damage response and cell cycle regulatory proteins such as p53 to prevent cell-cycle arrest. Using its origin DNA binding activity, Tag assembles as a double hexamer on the viral origin of replication, which serves two functions: inhibition of the early and activation of the late promoter for the transcription of viral structural proteins. Inhibition of its own transcription by Tag presumably optimizes Tag levels in the nucleus. Binding of Tag to SV40 origin DNA also triggers recruitment of the cellular replication machinery, such as replication protein A (RPA) and polymerase-α-primase (pol-prim), and leads to initiation of viral genome replication.

**The three amigos of the monopolymerase assay**

Taking advantage of a well-defined origin and the limited number of proteins that are required for viral DNA replication, a simple assay was developed that utilizes Tag, RPA, Topoisomerase I (Topo I), and pol-prim to study initiation of SV40 DNA replication *in vitro*. In its original form, the assay utilized soluble extract from virus-infected cells (Li and Kelly 1984). When Tag was added to the extract, viral DNA replication ensued. Ten protein complexes were identified from a series of crude extract fractionations and were shown to be necessary and sufficient for SV40 initiation (Murakami et al. 1986; Ishimi et al. 1988; Wold et al. 1989; Weinberg et al. 1990; Eki et al. 1992; Waga et al. 1994). These initiation proteins, their activity, and subunit composition are listed in Table 1. An even simpler assay to measure initiation of replication can be constructed that utilizes Tag, RPA, pol-prim, and Topo I to support SV40 replication *in vitro* (Figure 5).

**Table 1:** Cellular proteins required for SV40 DNA replication*

| Protein | Subunit Composition | Replication Activity |
|---|---|---|
| SV40 T antigen | 90 kDa | Origin binding/unwinding, helicase |
| Replication protein A | 70, 32, 14 kDa | Single-stranded DNA binding |
| DNA polymerase α | 180, 68, 58, 48 kDa | DNA polymerase, DNA primase |
| DNA polymerase δ | 125, 66, 50, 12 kDa | DNA polymerase, 3'-5' exonuclease |
| Proliferating Cell Nuclear Antigen | 37 kDa | Pol δ processivity factor |
| Replication factor C | 140, 40, 38, 37, 36 | PCNA clamp loader |
| Topoisomerase I | 100 kDa | Relieves torsional stress in DNA |
| Topoisomerase II | 140 kDa | Unlinks daughter duplexes, Relieves torsional stress in DNA |
| RNase H1 | 68 kDa | Endonuclease specific for RNA-DNA hybrid primers |
| FEN1 | 44 kDa | 5'-3' flap exonuclease |
| DNA ligase 1 | 125 kDa | Joins Okazaki fragments |

*Adapted from Herendeen and Kelly, 1996; Frontiers in Molecular Biology
Hurwitz et al., 1990. J. Biol Chem. 265: 18043-18046.
Waga and Stillman, 1998. Annu. Rev. Biochem. 67: 721-751.

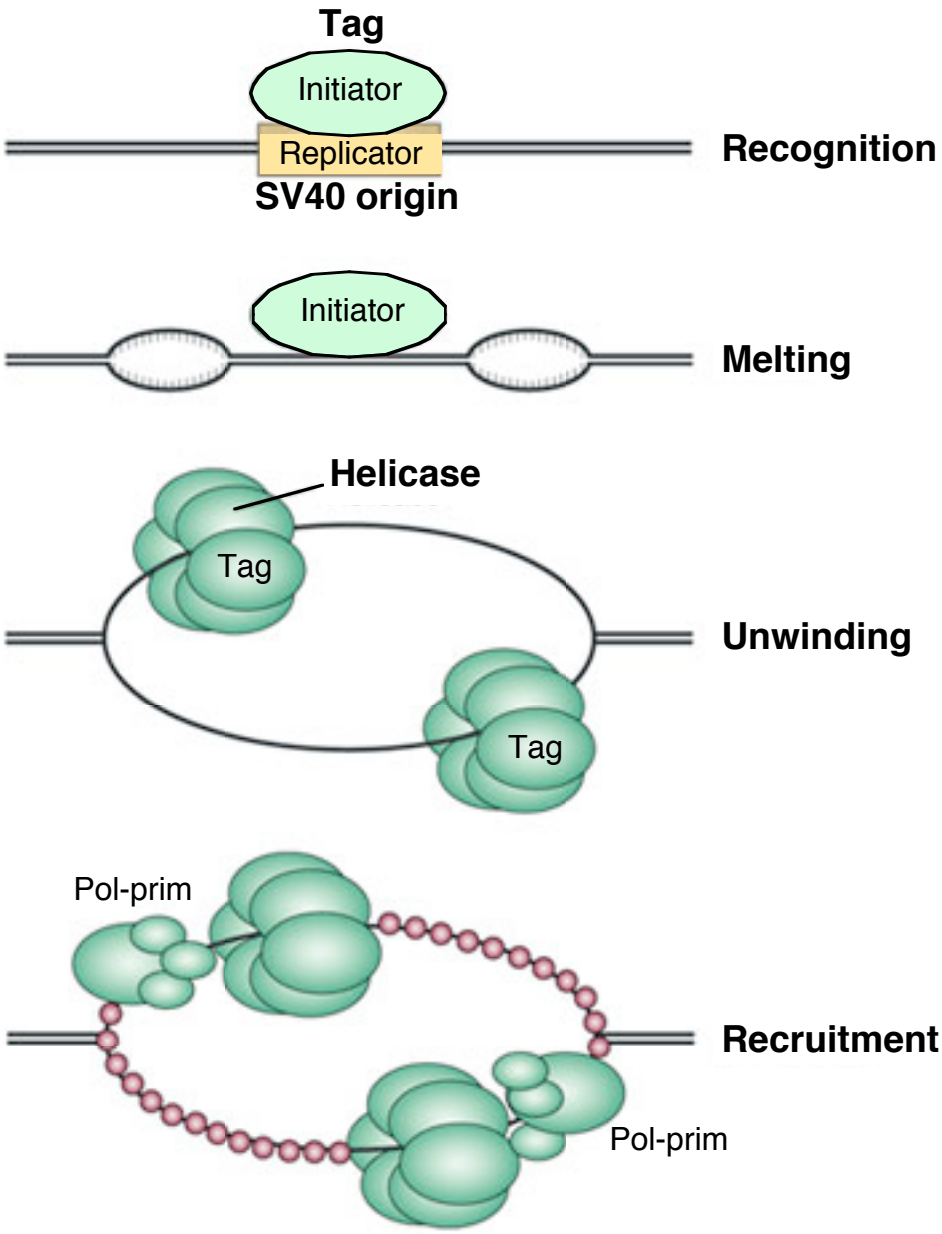


**Figure 5. A model for SV40 initiation**. Initiation of SV40 replication can be divided into four steps: recognition, melting, unwinding, and recruitment. All of these steps are mediated by the viral initiator protein Tag.

**Large T antigen as a master of ceremonies in SV40 replication**

SV40 Tag is a 708 amino acid peptide with a predicted molecular weight of 82 kDa. Several functional domains were identified in Tag and most were shown to be required for Tag function in DNA replication (DePamphilis and Bradley 1986). The N-terminal domain is usually defined from the initiation codon to the NLS (residues 1-125) and the first 75 residues form a functional domain very similar to the J-domain motif. This domain is dispensable for SV40 DNA replication *in vitro* (Tjian et al. 1980; Klausing et al. 1989; Weisshart et al. 1996) but not *in vivo* (Pipas et al. 1983; Campbell et al. 1997), and it contributes to the transforming function and carries out several mitogenic activities of Tag (Bullock 1997). In particular, the N-terminal domain interacts with a number of cell cycle regulatory and gene expression proteins of the host, such as Rb family members, hsc70 family, and pol-prim (Fanning and Knippers 1992).

Tag/DNA interactions are mediated through Tag's origin binding domain (OBD). A crystal structure of full length Tag could not be determined due to the failure to obtain crystals (Fanning and Knippers 1992), and a new approach to determine the structure of Tag's functional domains using NMR was utilized. Residues 131-259 were determined to constitute the OBD (Arthur et al. 1988) and the structure presented a novel DNA interaction fold (Luo et al. 1996).

The origin binding domain is considered central to Tag function and was proposed to coordinate other domain activities (Lin et al. 1992). Indeed, the Tag-OBD is sufficient to recognize and specifically bind to the SV40 replication origin (Peden and Pipas 1985; Paucha et al. 1986; Arthur et al. 1988; McVey et al. 1989; Hoss et al. 1990; Simmons et al. 1990b; Simmons et al. 1990a; Joo et al. 1997). It contains non-specific

dsDNA (Wun-Kim and Simmons 1990; Lin et al. 1992) and ssDNA binding activities (Wu et al. 2001). Initial studies defined the minimal OBD domain, which consists of residues 131-627 (Wun-Kim and Simmons 1990; Wu et al. 2001). However, structural studies indicated that a peptide containing residues 251-627 possesses helicase activity (Li et al. 2003). Tag is a hexameric helicase with a 3'→ 5' activity and belongs to the helicase superfamily III and is also a member of AAA+ proteins (Koonin 1993; Patel and Picha 2000). The helicase domain can be further sub-divided into ATPase (residues 418-616) (Giacherio and Hager 1979; Clark et al. 1983; Wun-Kim and Simmons 1990) and nucleotide-binding (residues 418-528) (Bradley et al. 1987) domains. ATPase activity is required for the initiation of DNA replication by Tag (Clark et al. 1983) and for the oligomerization of individual subunits into a double hexamer (Mastrangelo et al. 1989; Dean et al. 1992). Tag helicase activity does not require a free ssDNA tail to initiate replication (Borowiec and Hurwitz 1988) (Figure 6). A small 38 amino acid

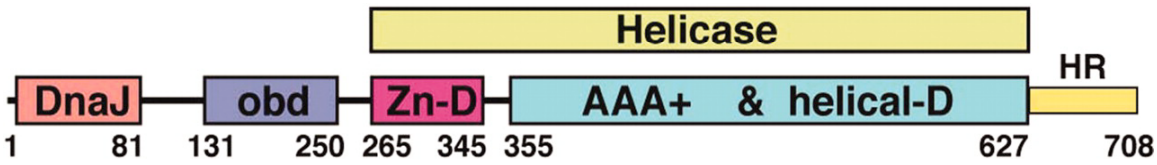


**Figure 6. Cartoon representation of the known Tag domain structures**. The amino acid numbers are indicated at the bottom. The functional domains are represented by open boxes and are labeled accordingly. The linkers between domains are represented by thin lines. The C-terminal domain from residue 628 to 708, which contains the host-range fragment (residues 682-708), is labeled HR (in yellow) for convenience. The HR domain is thought to be unstructured. The figure and the legend were adapted from Gai et al., 2004.

carboxyl-terminal domain of Tag is not required for DNA replication, but is important for the viral particle assembly (Pipas 1985; Khalili et al. 1988; Stacy et al. 1989) (Figure 6).

To fulfill its function in DNA replication, Tag interacts with a number of cellular replication proteins. Two pol-prim interaction sites were identified in Tag: a high-affinity binding site at residues 272-517 and a weaker site located within the first 82 amino acids (Smale and Tjian 1986; Gannon and Lane 1987; Schmieg and Simmons 1988; Dornreiter et al. 1990; Dornreiter et al. 1993; Weisshart et al. 1996). Topo I also has two interaction sites with Tag. One region resides between residues 82 and 246, while the other weaker site is located after residue 246 (Simmons et al. 1996). The human RPA binding site has been mapped to a region between residues 164-249, which overlaps with Tag-OBD (Dornreiter et al. 1992; Weisshart et al. 1998).

Tag also interacts with a number of cell cycle control proteins, including p53, Rb, and several Rb family proteins (Lane and Crawford 1979; Linzer and Levine 1979; DeCaprio et al. 1988; Dyson et al. 1989). The ability of Tag to bind and sequester p53 and Rb is thought to be crucial for Tag-mediated transformation effectiveness (Levine 1993), but is not required for its DNA replication activity (Lin and Simmons 1991).

Transcription proteins are also regulated by interactions with Tag. This group includes CREB binding protein (CBP) family members, such as CBP, p300, and p400, transcription factors AP2, TATA-binding protein (TBP), TFIID, TFIIB, and Transcription Enhancing Factor 1 (TEF-1) among other transcription regulatory elements (Mitchell et al. 1987; Gruda et al. 1993; Dickmanns et al. 1994; Berger et al. 1996; Johnston et al. 1996; Lill et al. 1997). Several other proteins with various functions were shown to interact with Tag (e.g. hsc70) (Campbell et al. 1997).

**DNA polymerase  - primase**

Pol-prim is a four-subunit complex that is unique among other DNA polymerases for also possessing a primase activity, which enables pol-prim to generate nascent DNA strands *de novo*. All four subunits of pol-prim are conserved in eukaryotes and are necessary for yeast cell viability (Sugino 1995). The tetramer initiates DNA replication by synthesizing short RNA primers on the leading and lagging strands and extending them with deoxynucleotides to create RNA/DNA hybrid chains of approximately 35 bp (Fioani et al. 1997; Waga and Stillman 1998; Arezi and Kuchta 2000; Hubscher et al. 2000).

The largest (180 kDa) subunit of pol-prim contains the DNA polymerase catalytic center and elongates the 8-12 nucleotide-long RNA primers, which are generated by pol-prim's primase activity (Plevani et al. 1985). Primase activity is associated with the p48 subunit (Santocanale et al. 1993), which exists in a tight complex with p58 (Copeland and Wang 1993). p58 might facilitate the primer synthesis of the primase and stabilize p48 association with the rest of the subunits, in particular p180 (Longhese et al. 1993). Some studies suggest that p58 might regulate p48 expression and nuclear transport by a "piggy-back" mechanism (Mizuno et al. 1996; Mizuno et al. 1998). No catalytic activity has been found for the p68 subunit (also termed subunit B) (Collins et al. 1993; Foiani et al. 1994). It was proposed that p68 might function as a tether to pol-prim and Tag at the origin of replication (Collins et al. 1993). Genetic studies in budding yeast suggest a role for subunit B in the early steps of DNA replication before dNTP polymerization (Foiani et al. 1994). A recent study, utilizing the SV40 *in vitro* replication system, placed the

function of p68 at the priming step of initiation in the presence of RPA and Tag and confirmed that it is required for primosome activity (Ott et al. 2002b).

Pol-prim mRNA levels remain constant during the cell cycle with only a slight increase prior to cell entry into the S phase. Periodic transcription is not required for DNA synthesis (Wahl et al. 1988; Falconi et al. 1993; Miyazawa et al. 1993). These observations suggest that post-translational modifications regulate pol-prim activity during the cell cycle. Indeed, both yeast and human pol-prim complexes are phosphorylated by CDKs in a cell cycle dependent manner (Nasheuer et al. 1991; Foiani et al. 1995; Ferrari et al. 1996). Phosphorylation of yeast p68 by CDKs was shown to occur at the $G_1$/S transition in yeast and human cells, and increases as cells progress to $G_2$/M (Nasheuer et al. 1991; Foiani et al. 1995; Ferrari et al. 1996; Voitenleitner et al. 1999; Dehde et al. 2001). Human p68 undergoes phosphorylation by Cyclin A/cdk2 and associates with the kinase and a phosphatase in human cells (Dehde et al. 2001; Ott et al. 2002b). Interestingly, dephosphorylation of the complex occurs after exit from mitosis and coincides with pre-RC formation (Diffley et al. 1994), while CDK phosphorylation of pol-prim renders it unable to initiate primer synthesis in SV40 DNA replication (Ott et al. 2002b).

**The many hats of RPA**

Replication protein A (RPA) was identified as a heterotrimeric single-stranded DNA (ssDNA) binding protein required for replication of simian virus 40 (SV40) DNA *in vitro* (reviews: (Borowiec et al. 1990; Fanning and Knippers 1992; Bullock 1997; Waga and Stillman 1998; Simmons 2000; Stenlund 2003). RPA is now known to be essential for

chromosomal DNA replication, repair, and recombination pathways in eukaryotic cells, and new roles in DNA damage signaling and regulation of replication origin firing frequency are emerging (Wold 1997; Iftode et al. 1999; Zou and Elledge 2003; Bochkarev and Bochkareva 2004; Shechter et al. 2004; Stauffer and Chazin 2004). RPA functions to protect ssDNA from nucleases and prevent hairpin formation in ssDNA that would interfere with DNA processing, but it also appears to actively coordinate the sequential assembly and disassembly of DNA processing proteins on ssDNA (Yuzhakov et al. 1999b; Kowalczykowski 2000). The ability of RPA to guide DNA processing depends on RPA interactions with other proteins in each pathway, but these mechanisms are not well understood.

RPA contains four ssDNA binding domains of different affinities, three in the 70-kDa subunit, connected to each other through flexible linkers, and one in the 32-kDa subunit. RPA binds to ssDNA in three different modes characterized by the length of ssDNA that it contacts (8-10, 19-23, and 28-30 nucleotides) and the number of ssDNA binding domains involved (Bastin-Shanower and Brill 2001; Bochkareva et al. 2001). Binding to ssDNA is thought to occur sequentially from 5' to 3', beginning with the RPA70 domains A and B in an initial 10 nucleotide binding mode and progressing to the high affinity 27-30 nucleotide binding mode involving all four ssDNA binding domains (de Laat et al. 1998; Iftode and Borowiec 2000; Bochkareva et al. 2001; Bochkareva et al. 2002; Arunkumar et al. 2003; Wyka et al. 2003). The three ssDNA-binding modes of heterotrimeric RPA imply that the protein could adopt three different structural conformations. Indeed scanning transmission electron micrographs demonstrate RPA molecules in compact and extended conformations on ssDNA (Blackwell et al. 1996).

Structures for all six globular domains of RPA have been determined (reviewed by (Bochkarev and Bochkareva 2004)).

The physical interactions of RPA with multiple DNA processing proteins have been mapped and some of these have been shown to be functionally required in the corresponding processing pathway. For example, human RPA70 residues 181-422 bind to SV40 T antigen in a region that overlaps the T antigen origin DNA binding domain (residues 164-249) (Dornreiter et al. 1992; Braun et al. 1997; Weisshart et al. 1998; Han et al. 1999). An anti-T antigen monoclonal antibody whose epitope maps in the RPA-binding domain specifically prevents physical interaction with RPA and inhibits SV40 DNA replication *in vitro* (Weisshart et al. 1998). Consistent with an essential role for specific interactions of human RPA with T antigen, yeast RPA fails to bind T antigen and to support SV40 replication (Melendy and Stillman 1993), although yeast RPA, like a number of other ssDNA-binding proteins, does support origin DNA unwinding (Kenny et al. 1989). The next step in initiation, T antigen-mediated primer synthesis on RPA-coated ssDNA by DNA polymerase a-primase (pol-prim), specifically requires human RPA, suggesting that T antigen association with RPA facilitates priming (Melendy and Stillman 1993; Wang et al. 2000). However, a detailed understanding of this process is still lacking.

The C-terminal domain of RPA32 (RPA32C), a winged helix-loop-helix, interacts

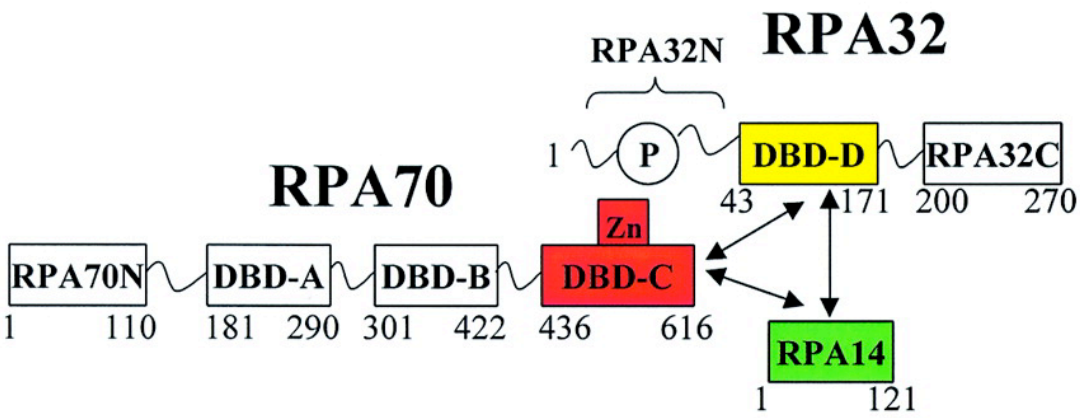


**Figure 7. Schematic showing the RPA domain structure.** Domains are presented as boxes, their borders are indicated. Zn, zinc ribbon; P, unstructured, phosphorylated N-terminus of subunit RPA32. Domains comprising the trimerization core are colored in red, yellow and green (for DBD-C, DBD-D and RPA14, respectively). The regions of subunit interaction are indicated by arrows. The figure and the legend were adapted from Bochkareva et al., 2002.

physically with the DNA repair and recombination proteins XPA, UDG2, and RAD52 (Mer et al. 2000b) (Figure 8). It is not yet known whether replication proteins also interact with RPA32C, but initial analysis of the interacting domains suggests similarity of the mechanisms (Arunkumar and Chazin, unpublished) (Figure 8). Multiple lines of evidence implicate RPA32 in SV40 DNA replication. Antibodies against RPA32 specifically inhibit SV40 replication *in vitro* (Kenny et al. 1990; Erdile et al. 1991). In the context of trimeric RPA, RPA32 can be directly cross-linked to nascent RNA-DNA primers (Mass et al. 1998) and the RPA trimerization core alone (RPA70C-RPA32D-RPA14) was recently shown to bind to a primer-template junction (Pestryakov et al. 2004). Binding of human RPA32 to T antigen has been reported (Lee and Kim 1995; Wang et al. 2000), but not confirmed by others (Braun et al. 1997; Han et al. 1999; Loo and Melendy 2004). Similarly, RPA mutants with deletions in the C-terminal domain of RPA32 supported SV40 replication poorly in one study, but displayed nearly wild type activity in another investigation (Lee and Kim 1995; Braun et al. 1997).

Due to discrepancy in RPA32C analysis, we decided to analyze the function of RPA32C in *in vitro* SV40 replication assay using recombinant human and viral proteins. We tested whether RPA32C is able to interact with Tag-OBD and what role, if any, these interactions play in the initiation of SV40 replication. Affinity chromatography and NMR studies allowed us to predict interacting domains of the two proteins and suggested the target residues for mutagenic analysis. Mutants that failed to interact with Tag-OBD were then tested in SV40 replication assays to assess their ability to support replication. A model for the role of RPA32C in initiation of SV40 replication was proposed based on the structural and biochemical data.

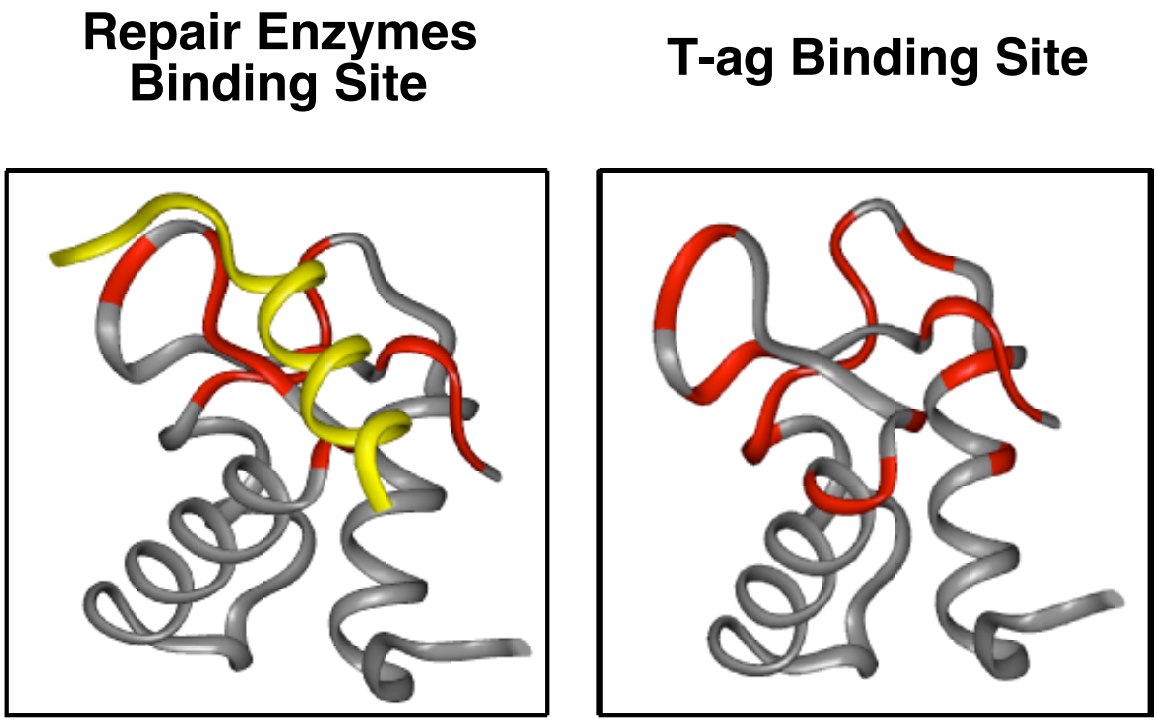


**Figure 8. RPA32C binding sites to Tag and repair enzymes.** A ribbon model of hRPA32C. Interacting residues are shown in red. Yellow helix represents interacting domain of XPA, UNG2, Rad52.

CHAPTER II

# THE CARBOXYL-TERMINAL DOMAIN OF THE 32-KDA SUBUNIT OF REPLICATION PROTEIN A IS REQUIRED FOR SV40 DNA REPLICATION[1]

## Introduction

The fundamental biochemical steps in eukaryotic DNA replication were first elucidated in studies of a simple but powerful model system, the cell-free replication of the simian virus 40 (SV40) genome. A single viral protein, large T antigen (Tag), orchestrates the entire replication process in primate cell extracts. Tag directs the initiation of viral replication by specifically binding to the SV40 origin of DNA replication, assembling into a double hexameric helicase that unwinds the duplex DNA bidirectionally, and recruiting cellular initiation proteins (Fanning and Knippers 1992; Bullock 1997; Simmons 2000; Stenlund 2003). The progression of SV40 replication requires a ssDNA-binding protein, replication protein A (RPA), which binds to the free ssDNA generated by the Tag helicase, and together with Tag, enables primer synthesis and extension by DNA polymerase alpha-primase (pol-prim). The host replication machinery carries out all subsequent steps.

The molecular mechanism for the coordinated activities of Tag and human RPA (hRPA) has yet to be elucidated. Bacterial single-stranded DNA-binding (SSB) protein and RPA from budding yeast can support SV40 origin-specific DNA unwinding by Tag, but not primer synthesis and extension (Bullock 1997). The activity of hRPA in the

[1] Arunkumar, A*., Klimovich, V*., Jiang, X., Ott, R., Mizoue, L., Fanning, E., and Chazin, W. (2005). Structural and functional insights into RPA32 C-terminal domain-mediated assembly of the simian virus 40 replisome. , Nat. Struc. Mol. Biol., in press *These authors contributed equally to this work.

initiation of viral DNA replication correlates well with its ability to interact physically with Tag, while SSBs that support unwinding but not initiation bind poorly to Tag (Collins and Kelly 1991; Dornreiter et al. 1992; Melendy and Stillman 1993). These results and other genetic and biochemical data strongly suggest that direct physical interactions between Tag and RPA are crucial for initiation of SV40 replication.

It has become increasingly apparent that DNA processing events involve modular proteins that contain multiple structural/functional domains and have multiple points of contact (Stauffer and Chazin 2004). Tag and RPA are both modular proteins (Bullock 1997; Wold 1997; Iftode et al. 1999; Mer et al. 2000a; Bochkarev and Bochkareva 2004; Gai et al. 2004; Weisshart et al. 2004) but mapping of their interaction sites is incomplete. The Tag origin DNA-binding domain (Tag-OBD) has been identified as an RPA interacting site (Weisshart et al. 1998), and the 70 kDa subunit of RPA was shown to be involved in Tag interactions (Braun et al. 1997). The C-terminal domain of the 32 kDa subunit (RPA32C), a winged helix-loop-helix, is a known protein interaction module (Mer et al. 2000b), but its role in Tag interactions as well as in SV40 replication has been controversial (Lee and Kim 1995; Braun et al. 1997). Here, we demonstrate that RPA32C does indeed interact with Tag and in fact, plays a critical role in stimulating the initiation of SV40 replication. These findings show that the interaction between Tag and RPA involves multiple contact points, a critical feature that we incorporate into a refined mechanistic model for primer synthesis during SV40 replication.

## Materials and methods

### *Protein preparation*

Human RPA32C was expressed and purified as described (Mer et al. 2000b), except the final reversed phase high performance liquid chromatography (HPLC) step was replaced by a gel filtration column using HiLoad 16/60 Superdex 75 (Amersham Pharmacia). Yeast RPA32C domain was cloned into the same vector (pET15b), expressed in BL21 (DE3) cells, and purified in a similar manner as hRPA32C. Point mutants were generated by QuickChange (Stratagene) site-directed mutagenesis following vendor protocols, including: RPA32C E252A, E252R, Y256A, S257A, T267A, D268A and D268R; Tag R154A and R154E.

Recombinant RPA heterotrimers were expressed in *E. coli* and purified as described (Henricksen et al. 1994). Single amino acid substitutions in RPA32 of the hRPA heterotrimer were introduced by QuikChange.

*Construction of the RPA chimera hRPAy32C*

The amino terminal coding sequence of the human RPA32 subunit in pET11d-tRPA was amplified by PCR using the primers 5'-GGAACAGTGGATTCGAAAGC-3' and 5'-TTTGCTTAGTACCATGTGTG-3'. The carboxyl terminus of the budding yeast RPA32 subunit was amplified in a separate PCR reaction using the primers 5'-ACACATGGTACTAAGCAAATGTCATTCCATAG-3' and 5'-CGTAGGTACCTCATAGGGCAAAGAAGTTATTC-3'. The products from each PCR reaction were analyzed by agarose gel electrophoresis and the appropriate DNA products were excised and purified using the Qiagen (Valencia, CA) PCR purification kit. The

purified DNA products were then used as templates for the final PCR reaction with the primers 5'-GGAACAGTGGATTCGAAAGC-3' and 5'-CGTAGGTACCTCATAGGGCAAAGAAGTTATTC-3'. The PCR products were analyzed by agarose gel electrophoresis and the appropriate DNA products were excised and purified using the Qiagen PCR purification kit. The plasmid pET11d-tRPA encoding the three subunits of human RPA was simultaneously digested with Dra III/Kpn I. The PCR products were also digested in parallel with the same enzymes. The digest products were analyzed by agarose gel electrophoresis and the appropriate bands were purified as described above. The Dra III/ Kpn I vector fragment and the Dra III/ Kpn I PCR product were ligated and the reaction products were transformed into competent HB101 E. coli cells. Ampicillin-resistant clones were analyzed by restriction enzyme digestion and by DNA sequencing with the primer 5'-GCAGAGAAGGCTCCAACCAAC-3'. The RPA chimera protein, containing yeast residues 173-273 in place of human residues 172-270, was expressed and purified from *E. coli* as described (Henricksen et al. 1994).

*Construction of the S257P mutant of hRPA32*

An 881-bp Kpn I fragment containing the RPA32 coding sequence from nt 8382 to nt 9263 was excised from the hRPA expression plasmid pET11d-tRPA (Henricksen et al. 1994) and cloned into the Kpn I site of pBluescript KS II+ plasmid. The resulting plasmid DNA was used as the template for site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA) according to the manufacturer's protocol with the primers 5'-GGGGCACATCTATCCTACTGTGGATGATGACC-3' and 5'-GGTCATCCACAGTAGGATAGATGTGCCCC-3'. The mutant fragment was

completely sequenced and then cloned back into the Kpn I site of pET11d-tRPA for protein expression as described for hRPA.

SV40 Tag, topoisomerase I, and pol-prim were purified as described (Ott et al. 2002a).

$Tag_{131-259}$ (Tag-OBD) was cloned into an in-house pSV278 expression vector, which contains a 6x His tag followed by an N-terminal MBP fusion and a thrombin cleavage site before the insert. The fusion protein was purified over Ni-NTA. After thrombin cleavage and another passage over Ni-NTA, the protein was further purified over MonoS 10/10 and HiLoad 16/60 Superdex 75 (Amersham Pharmacia).

Uniformly enriched $^{15}N$ and $^{13}C,^{15}N$ samples were prepared in minimal medium containing 1 g/L $^{15}NH_4Cl$ (CIL, Inc.) and 2 g/L unlabeled or [$^{13}C_6$] glucose (CIL, Inc.), respectively. The DNA duplex [d(GCAGAGGCCGA).d(TCGATTCTTGC)] was purchased from Midland Certified Co. and used without further purification.

***NMR spectroscopy***

All NMR samples were concentrated to 100 mM in a buffer containing 2 mM DTT, 5 mM MgCl2, and 20 mM Tris-d11 at pH 7.0. NMR experiments were performed at 25 ºC using a Bruker AVANCE 600 MHz NMR spectrometer equipped with a single axis z-gradient Cryoprobe. Two-dimensional, gradient-enhanced $^{15}N$-$^{1}H$ HSQC and TROSY-HSQC spectra were recorded with 4K complex data points in the $^{1}H$ and 200 complex points in $^{15}N$ dimension. The $^{13}C$-$^{1}H$ HSQC spectra were acquired with 4096 x 600 complex data points. Attempts to obtain NOE distance constraints for the intermolecular interface by acquiring $^{13}C,^{1}H$-filter, edited spectra were unsuccessful, presumably due to

the intrinsically low sensitivity of the experiments and the relatively short lifetime of the complex. Residual dipolar couplings ($D_{NH}$) were measured using the strain-induced gel alignment procedure (Sass et al. 2000; Tycko et al. 2000). Briefly, a 4% polyacrylamide gel with an inner diameter of 6 mm was soaked with a solution of the complex containing $^{15}$N-enriched RPA32C or Tag-OBD with unlabeled partner protein at a molar ratio of 1:3 (labeled:unlabeled). After soaking for 24 hours at 4° C, the sample was stretched into the NMR tube using the funnel like device described by Bax (Bax 2003). Residual dipolar couplings were determined from the difference between one bond $^{15}$N-$^{1}$H splittings ($^{1}J_{NH}$ + $^{1}D_{NH}$) measured in the absence and presence of alignment media. The splittings were measured using a combination of HSQC and TROSY spectra. A total of 65 useable $^{1}D_{NH}$ values were obtained, 28 from RPA32C and 37 from Tag-OBD. Back-calculation of residual dipolar couplings from the structure of the complex was carried out using the program PALES (Zweckstetter and Bax 2000). NMR data were processed using XWINMR (Bruker) and analyzed using either FELIX2000 (Accelrys, Inc.) or Sparky (Goddard and Kneller). To determine $K_d$ for the interaction between RPA32C and Tag-OBD, a series of spectra were acquired after addition of unlabeled RPA32C into a 100 mM solution of $^{15}$N-labeled Tag- OBD. Additions were made such that 8 to 12 HSQC spectra were recorded starting with a molar ratio of 1:0 (labeled:unlabeled) up to a ratio of 1:10. The pH of the sample after each addition was monitored and corrected if necessary. Changes in amide proton and amide nitrogen chemical shifts of T199 and H203 were fit to a standard single-site binding equation as described previously (Arunkumar et al. 2003).

***Structure calculations***

The structure of the complex was modeled using HADDOCK (Dominguez et al. 2003) run on a home-built Linux cluster. The chemical shift perturbation data along with the solvent accessibility of the interacting side chains (calculated using the program NACCES; http://wolf.bms.umist.ac.uk/naccess) was used to obtain a set of ambiguous interaction restraints (AIRs) as described in the HADDOCK manual. The target distance of these constraints was set to 3.0 Å and all other parameters were set to the default values. A rigid body docking procedure was used to obtain 1500 conformers of the RPA32C-Tag complex using only the AIRs, van der Waal's energy and electrostatic terms using the program CNS (Brunger et al. 1998). The 200 best conformers based on the intermolecular energy were subsequently used for semi-flexible simulated annealing followed by refinement using explicit water. Residues 249-257, 259-262 and 266-270 of RPA32C, and 152-156, 181-182, 199-204 and 255-258 of Tag-OBD were allowed to be flexible in all stages of the docking procedure. Cluster analysis using an RMSD cut-off of 1.5 Å revealed two clusters, with 189 conformers in one and only 4 conformers in the other. The structure of the complex is represented by an ensemble of 20 conformers with lowest energy. The single representative structure (with the lowest energy) was used to back calculate $^{1}D_{NH}$ for comparison to the experimental values. Structures were visualized and figures were generated using MOLMOL (Koradi et al. 1996).

***SV40 DNA replication (monopolymerase) assay***

A published protocol (Matsumoto et al. 1990) was modified as follows: reaction mixtures (20 $\mu$l) contained 250 ng of supercoiled pUC-HS plasmid DNA (2.8 kb)

containing the complete SV40 origin (Ott et al. 2002a), 200 ng of RPA, 300 ng of topoisomerase I, 100 to 400 ng of pol-prim as indicated in the figure legends, and 250-750 ng of Tag in initiation buffer (30 mM Hepes-KOH (pH 7.9), 7 mM magnesium acetate, 10 mM $ZnCl_2$, 1 mM DTT, 4 mM ATP, 0.2 mM each GTP, UTP, and CTP, 0.1 mM each dGTP, dATP, and dCTP, 0.02 mM dTTP, 40 mM creatine phosphate, 40 g/ml of creatine kinase supplemented with 3 $\mu$Ci of [-$^{32}$P] dTTP (3,000 Ci/mmol; Dupont NEN, Boston, MA). Reactions were carried out and results evaluated as described previously (Ott et al. 2002a). Primer synthesis reactions were identical except that 20 $\mu$Ci of [-$^{32}$P] CTP (3,000 Ci/mmol; Dupont NEN) was the labeled nucleotide and the dNTPs and the ATP regenerating system were omitted. Products were analyzed as described (Ott et al. 2002a).

***T antigen-dependent primer synthesis and extension assays on ssDNA***

The reaction mixture was identical to that in the SV40 monopolymerase assay except that the template was generally 100 ng of M13mp18 ssDNA (USB Corp., Cleveland, OH) that had been pre-incubated for 20 min on ice with a saturating amount of RPA. After the pre-incubation, the remaining components were added and the assay was completed as described for the monopolymerase assay. Singly primed template ssDNA was prepared by mixing 4.2 pmol each of M13mp18 and a 17-mer sequencing primer (-40 primer, USB Corp.), heating at 60 ºC for 2 min and annealing at room temperature. The product was purified by 1% agarose gel electrophoresis and extracted from the gel using a kit (Qiagen). The primer extension reaction was carried out as described above, except that

the singly primed ssDNA was substituted for unprimed ssDNA in the pre-incubation with RPA, and that ribonucleotides, Tag, creatine phosphate, and creatine kinase were omitted.

## Results

### *An RPA32C antibody inhibits initiation of SV40 replication*

Our studies were initiated based on the observation that an antibody against RPA32 (Ab34A) specifically inhibited SV40 DNA replication in crude extracts in vitro (Kenny et al. 1990). Ab34A has little effect on the ssDNA binding activity of RPA, its ability to support origin DNA unwinding, or to stimulate DNA polymerase delta activity, but it does inhibit RPA stimulation of DNA polymerase alpha activity (Kenny et al. 1990). To map the epitope recognized by Ab34A, we purified human and yeast RPA (hRPA and yRPA), as well as hRPA carrying mutations in RPA32 (Figure 9a, b), and tested for their recognition by Ab34A in western blots (Figure 9c). Ab34A detected hRPA32 (Figure 9c, lane 1), but not yRPA32 (lane 2). Human RPA32C alone (residues 172-270) was sufficient to bind Ab34A (lane 3), but an hRPA chimera with hRPA32C substituted by the yeast domain (hRPAy32C) did not bind Ab34A (lane 4). Moreover, deletions of 33 or 48 residues from the C-terminus of hRPA32, which are predicted to destroy the globular structural domain of RPA32C, also prevented binding of Ab34A (lanes 5, 6). These data suggest that the antibody recognizes an epitope in the winged helix-loop-helix domain of RPA32.

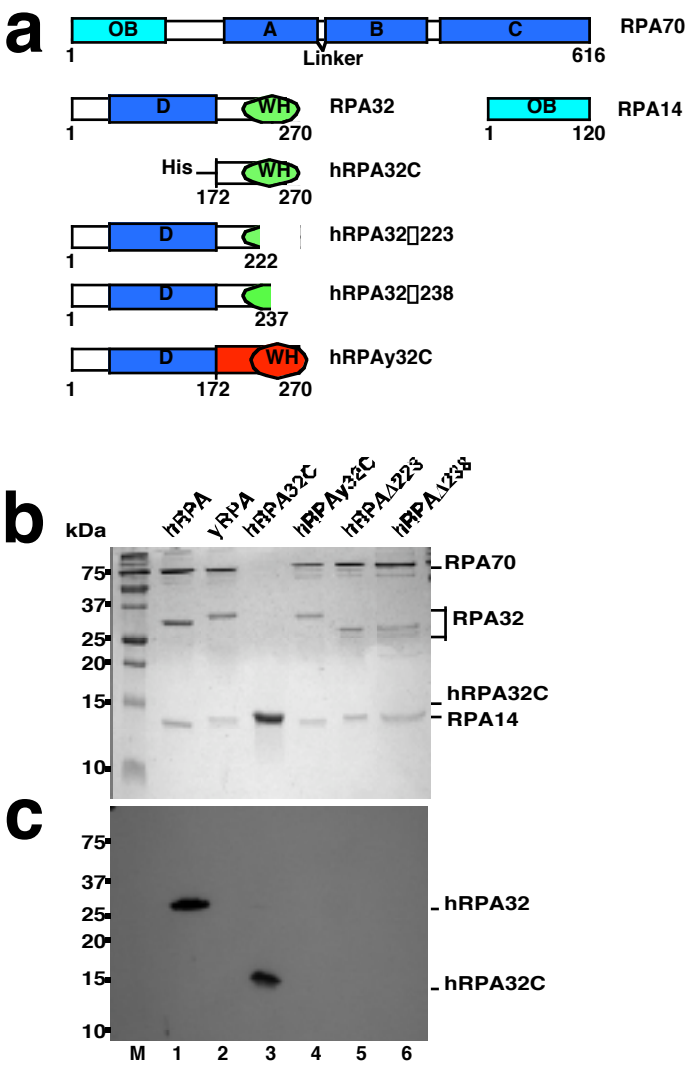


**Figure 9. Monoclonal antibody 34A recognizes hRPA32C.** (**a**) RPA subunits and mutant proteins used in this study are depicted schematically. Amino acid residue numbers are listed below each construct. OB, oligonucleotide-oligosaccharide binding folds, including the ssDNA binding domains A, B, C, and D; WH, winged helix-loop-helix domain. (**b**) Purified recombinant RPA proteins were analyzed by 15% SDS-PAGE and Coomassie staining. M, protein markers of the indicated mass in kDa. (**c**) Western blot assay of the proteins shown in panel b, probed with 34A monoclonal antibody and visualized by chemiluminescence.

To confirm that Ab34A inhibits initiation of SV40 DNA replication, monopolymerase reaction assays (Matsumoto et al. 1990) were performed using purified proteins. In this assay, synthesis of radiolabeled DNA depends on Tag assembly on the SV40 origin DNA, unwinding of the duplex, and synthesis of RNA primers that can then be extended. Radiolabeled products were analyzed by denaturing gel electrophoresis, followed by autoradiography panel a. Robust DNA replication occurred in a positive control reaction carried out in the absence of antibody (Figure 10a, lane 12). Negative control reactions yielded no detectable products (Figure 10, lanes 4, 5 and 9-11). Addition of Ab34A inhibited initiation in a dose-dependent manner, but the presence of a non-immune control antibody had no effect (Figure 10a, compare lanes 1-3 with 6-8). Quantified data of a typical reaction is presented in Figure 10b.

Previous studies with Ab34A indicated that the antibody did not interfere with origin DNA unwinding (Kenny et al. 1990), suggesting that it might inhibit the subsequent primer synthesis and elongation steps in initiation. To determine if RPA32C is required for these processes, ssDNA saturated with hRPA was used as a template for synthesis of unlabeled primers and extension into radiolabeled DNA products by pol-prim. Priming is inhibited on ssDNA saturated with RPA, but in the presence of Tag, pol-prim assembles into a functional primosome capable of primer synthesis on the RPA-ssDNA template (Matsumoto et al. 1990; Collins and Kelly 1991; Melendy and Stillman 1993). As expected, robust primer synthesis and elongation was observed on naked ssDNA template (Figure 10c, lane 1), but when the ssDNA was saturated with RPA, little synthesis was detected (lanes 3, 4, 7, 8). Addition of Tag stimulated priming

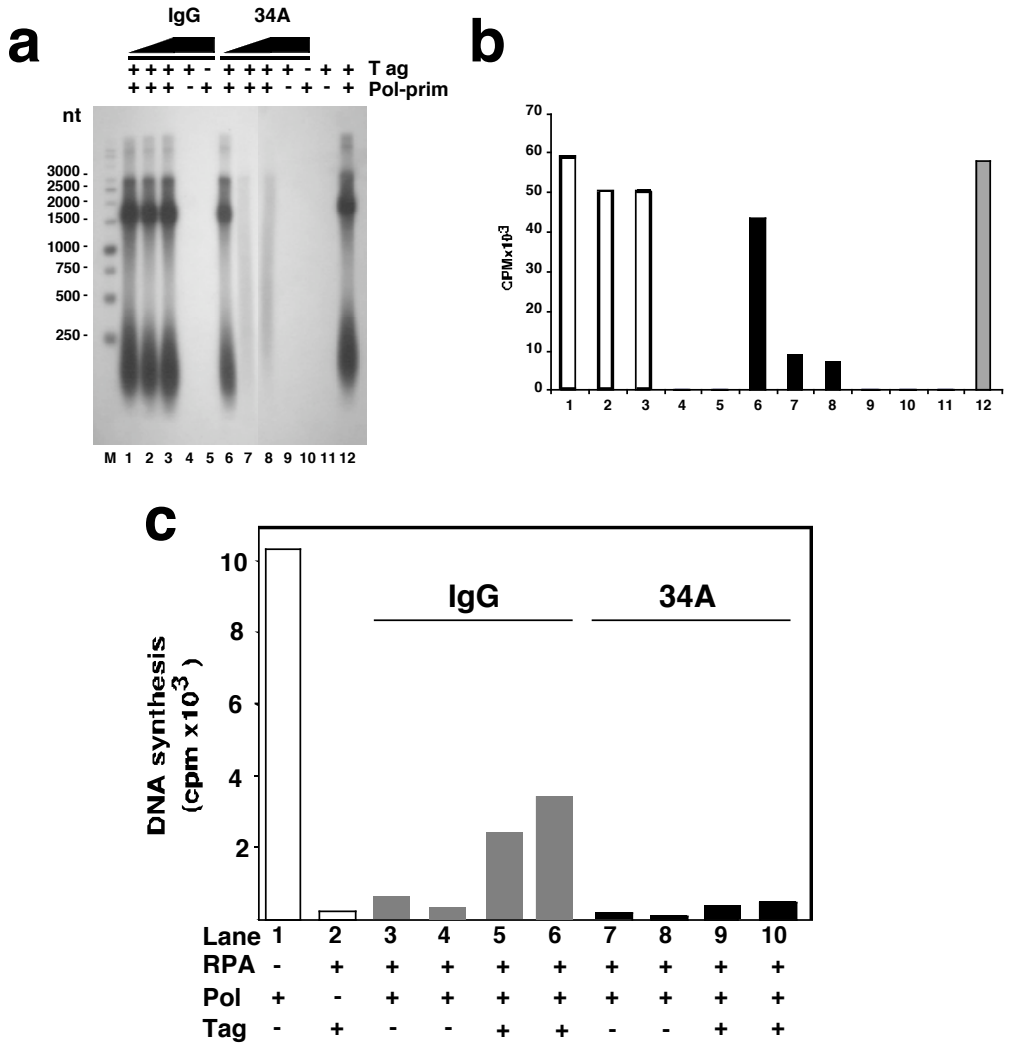


**Figure 10**. **Anti-RPA32 monoclonal antibody inhibits SV40 replication**. (**a**) Ab34A IgG or non-immune mouse IgG was titrated into SV40 monopolymerase assays reconstituted with purified recombinant proteins. Products were resolved by alkaline agarose gel electrophoresis and visualized by autoradiography. Lanes 1-3 and 6-8, reactions containing 100, 300, or 500 ng of the indicated antibody. Control reactions carried out in the presence of 500 ng of antibody and in the absence of T antigen or pol-prim are indicated (-). M, DNA size marker of the indicated length in nucleotides (nt). (**b**) Quantification of the reaction in panel a by scintillation counting. DNA synthesis is expressed in counts per minute (cpm.) (**c**) Primer synthesis and extension on M13mp18 ssDNA (25 ng) pre-incubated with 500 ng (lanes 3, 5, 7, 9) or 750 ng (lanes 2, 4, 6, 8, 10) of RPA was tested in the presence of 250 ng pol-prim and 500 ng of either non-immune IgG (lanes 3-6) or Ab34A (lanes 7-10). Control reactions with pol-prim alone (lane 1) and in the absence of pol-prim (lane 2) are indicated (-).

and elongation in the presence of the non-immune control antibody (compare lanes 5/6 with 3/4). However, in the presence of Ab34A, Tag failed to stimulate priming and elongation (compare lanes 9/10 with 5/6). Hence, Ab34A interferes with the ability of Tag to mediate priming and elongation by pol-prim. Together, these data suggest a possible physical interaction between Tag and hRPA32C that facilitates priming and extension.

***RPA mutants truncated at RPA32 C-terminus are defective in SV40 replication***

The ability of Ab34A to inhibit SV40 replication suggested a functional importance of this domain in the ability of RPA to support replication. In order to further assess this observation, a truncation mutant lacking 43 residues at the extreme C-terminus was generated (Figure 9a) and was shown to be severely defective in SV40 replication (Figure 11). As expected, human RPA vigorously supported Tag-stimulated DNA synthesis in the SV40 monopolymerase reaction (Figure 11a, lanes 1-4). On the contrary, the deletion mutant exhibited a severe defect in this reaction (lanes 5-8). The results support the hypothesis that RPA32C is necessary for Tag-mediated nascent DNA synthesis. Quantification of the results by scintillation counting is presented in Figure 11b.

The SV40 monopolymerase reaction does not differentiate whether the mutant-induced defect occurs at origin unwinding or at primer synthesis and elongation. Additional insight into distinguishing these possibilities could be gained by using an M13-derived ssDNA-based replication assay. This system does not involve Tag origin binding and unwinding. The results (Figure 11c) suggest that the observed defect occurs

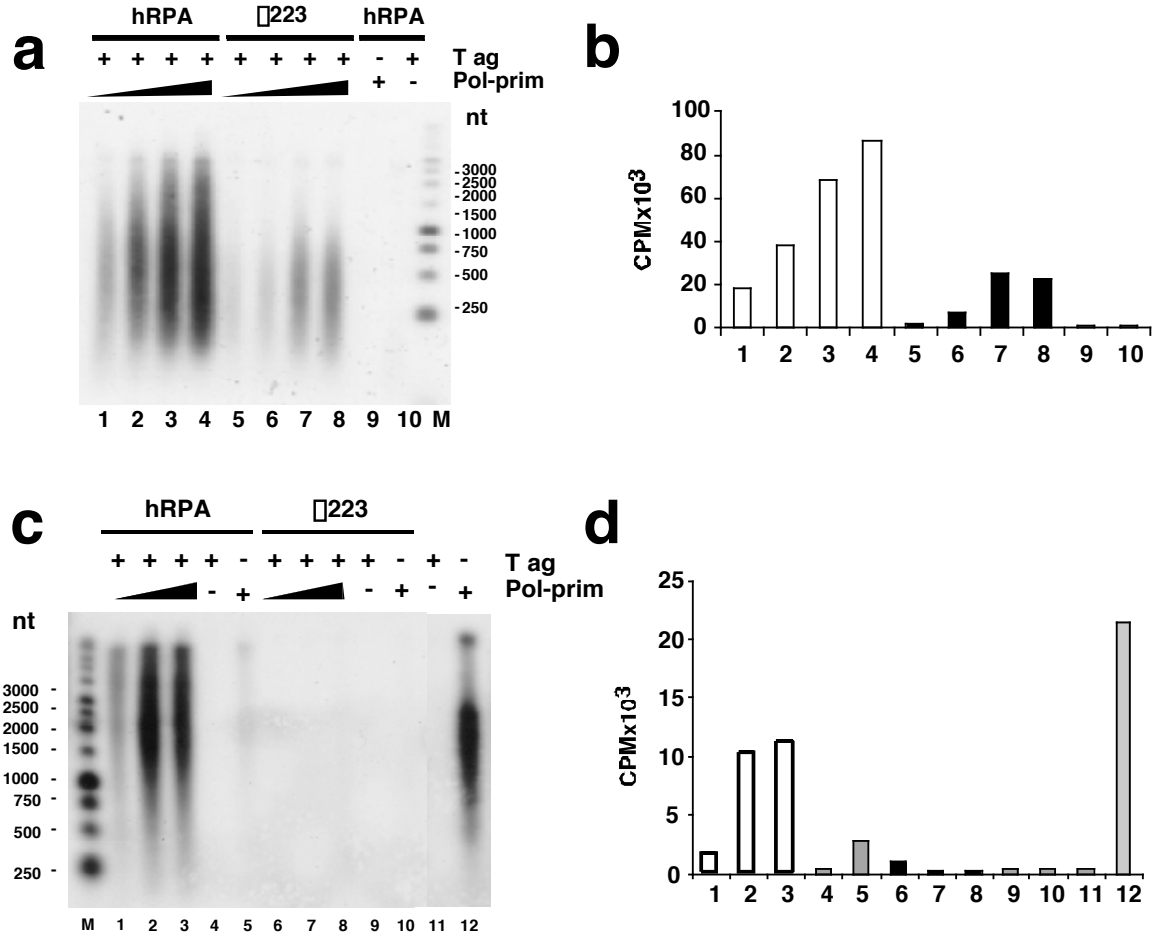


**Figure 11. RPA with a deletion in RPA32 C-terminus is defective in SV40 DNA replication**. (**a**) Monopolymerase reaction using 100 to 400 ng of pol-prim was conducted, DNA products were resolved on alkaline agarose gel and visualized by autoradiography. Negative control in the absence of Tag or pol-prim are indicated (-). DNA size markers are indicated (M). (**b**) Quantification of the results shown in panel a using scintillation counter. (**c**) Primer synthesis and elongation was assayed using M13 ssDNA and 100 to 300 ng of pol-prim. The DNA products were resolved and visualized as in panel a. (**d**) Quantification of the results in panel c using scintillation counter.

at the primer synthesis step of the initiation of DNA replication. Pol-prim was not able to initiate primer synthesis and elongation on ssDNA coated with saturating amounts of wt or mutant RPA (lanes 5 and 10). No product was generated in the absence of pol-prim (lanes 4, 9, 11), while abundant nascent DNA was produced on non-coated ssDNA in the presence of pol-prim (lane 12). Upon addition of increasing amounts of wt Tag, the RPA induced inhibition of replication was reversed in a dose dependent manner in the case of wt RPA (lanes 1-3), but not mutant RPA (lanes 6-8). These data strongly argue for a possible interaction between Tag and RPA32C in order to initiate primer synthesis and elongation.

***Biochemical characterization of hRPAy32C in SV40 replication***

The inability of RPA truncation mutants to support replication could be attributed to misfolding of the C-terminus of the protein due to disruption of important structural domains by the mutation. Since yRPA can support origin unwinding but not replication and has an overall similar domain structure like that of human RPA, we decided to create a mutant in which human RPA32C is replaced with a yeast sequence (Figure 9). The protein was expressed stably, and biochemical analysis revealed that it interacted with ssDNA and Tag with efficiency similar to wt hRPA (Figure 12). Despite its wt activity of ssDNA and Tag interaction, the chimeric protein had a severe defect in supporting SV40 replication (more detail in Figure 24 and Figure 25).

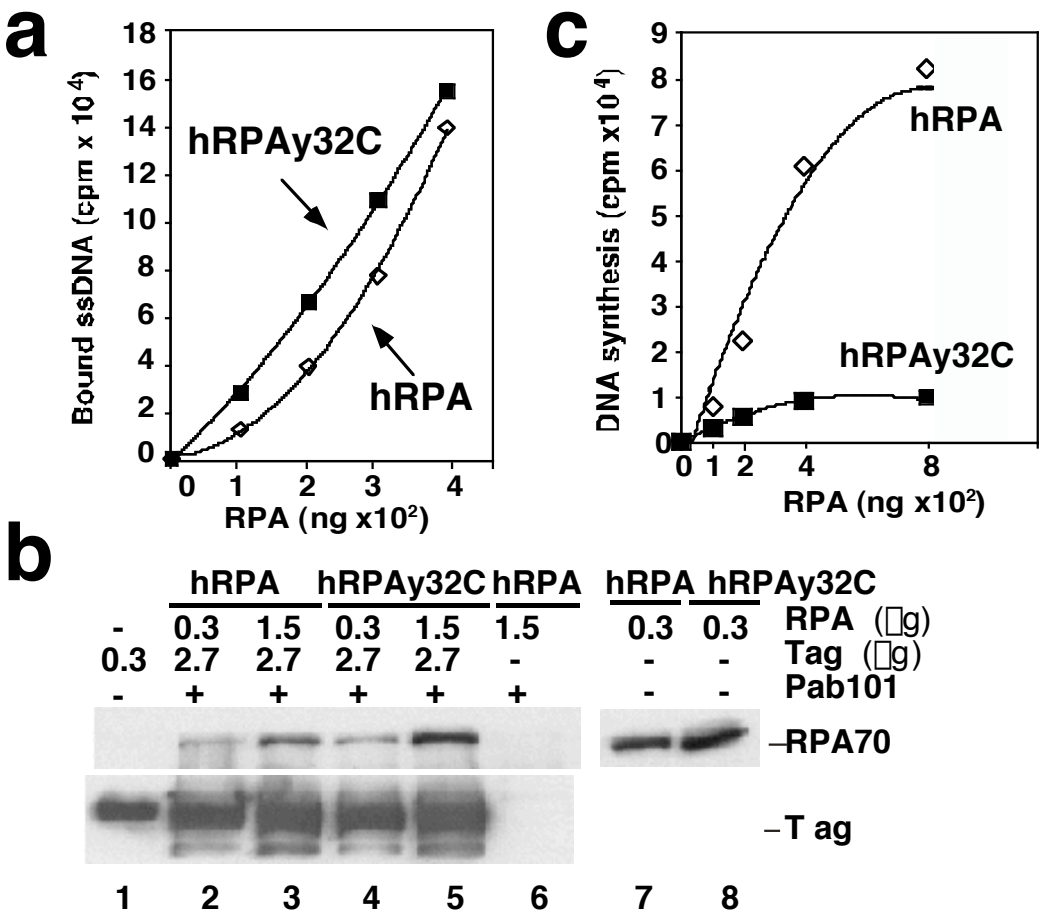


**Figure 12. Characterization of the hRPA32C and hRPAy32C mutant proteins.** (**a**) The indicated amounts of hRPA or hRPAy32C were incubated with 2.5 pmol of $^{32}$P-end-labeled oligo(dT)$_{30}$ and bound DNA was quantified by filter-binding and scintillation counting. Diamonds, hRPA; filled squares, hRPAy32C. (**b**) The indicated amounts of purified hRPA or hRPAy32C were incubated with or without (-) Tag bound to Pab101-coupled Sepharose beads for 30 min at 4ºC. After washing, proteins bound to the beads were analyzed by SDS-PAGE and western blotting with antibody against RPA70 (top panel) or Tag (bottom panel). Lanes 1, 7, 8: samples of the input proteins. (**c**) Initiation activity was tested as in Figure 4b except that the amount of hRPA or hRPAy32C was varied as indicated and 200 ng of pol-prim was present in each reaction. Acid-insoluble radiolabeled products in 4 μl of each reaction mix were quantified by scintillation counting. Diamonds, hRPA; filled squares, hRPAy32C. (Data in Figure 12 is from X. Jiang, Fanning lab.)

***A point mutation in RPA32C affects its activity***

Comparative analysis of human and yeast amino acid sequences provided an opportunity for the generation of mutants in which non-conserved human residues were replaced with yeast sequences (Figure 13a). We hypothesized that RPA32C/Tag interaction is compromised in the deletion mutants; therefore several mutants were created and analyzed for their ability to interact with Tag-OBD in a Far-Western assay (Figure 13b). A single point mutant S257P showed a marked defect in its ability to interact with Tag and was chosen for further analysis. We speculated that introducing a proline residue might create a slight shift in overall C-terminal domain orientation of RPA and affect its replication activity. As expected, the mutants exhibited a mild defect in SV40 replication compared to the wild type (Figure 13c, compare lanes 1-4 to 5-8). Due to the nature of serine to proline mutation that could cause misfolding of peptide, a circular dichroism analysis was conducted to verify the proper folding of this mutant (data not shown). Quantification of the autoradiograph in panel c is shown in panel d. On average the mutant had ~84% activity of the wild type (Figure 13e).

***RPA32C interacts specifically with the Tag-OBD***

In order to test for direct interaction between Tag and hRPA32C, affinity chromatography experiments were performed. Initial experiments suggested an interaction with the Tag-OBD. To confirm this observation, the Tag-OBD was passed

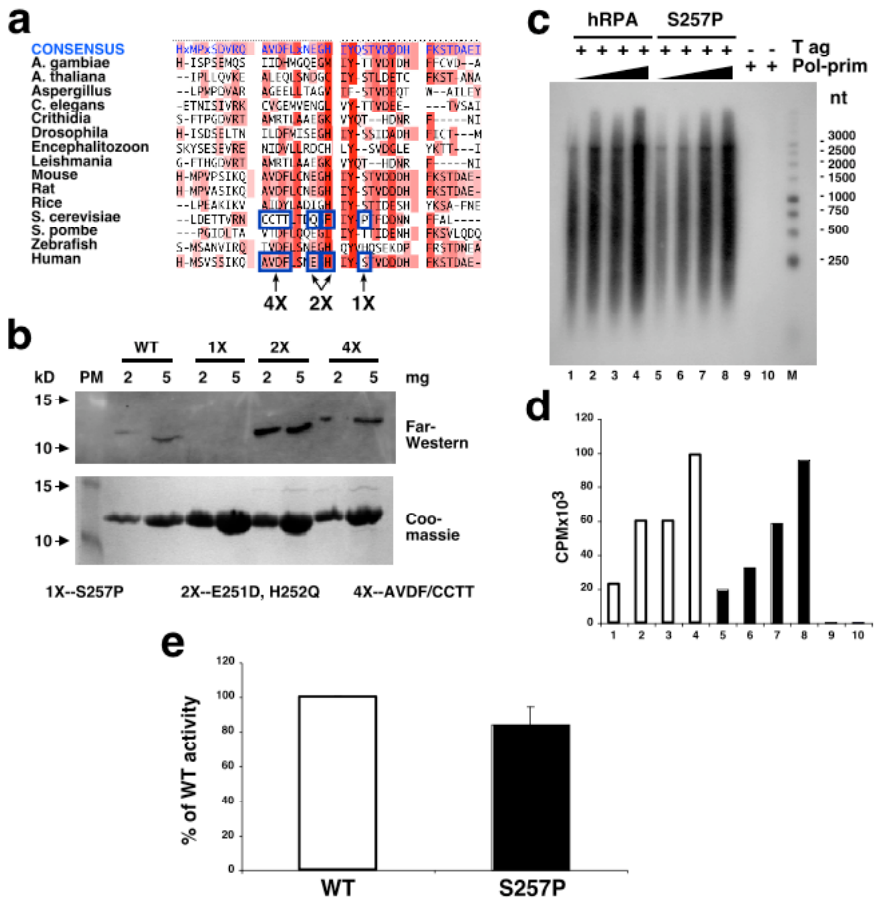


**Figure 13. A single point mutation affecting Tag interaction exhibits a defect in SV40 DNA replication.** (**a**) Multispecies alignment of RPA32C amino acid sequence. Human residue and yeast counterpart that replaced it in the mutant are indicated. (**b**) Far-Western analysis of selected mutants in RPA32C that substitute human sequences with the corresponding yeast residues indicated inability of S257P mutant to interact with Tag. The mutants of RPA32C were expressed as His-RPA32C protein, resolved on 15% SDS-PAGE, transferred onto nitrocellulose membrane, and overlaid with Tag. After extensive wash, the interacting proteins were probed with □-Tag polyclonal rabbit Ab and visualized by chemiluminescence. (**c**) SV40 monopolymerase reaction was conducted with 100-400 ng of pol-prim. The reaction products were resolved on alkaline agarose gel and visualized by autoradiography. Negative controls lacking Tag or pol-prim are indicated (-). DNA marker is labeled (M). (**d**) Quantification of results in panel c as assessed by scintillation counting. (**e**) Activity of S257P in SV40 replication was plotted to wt activity in the same experiment. Results represent at least two independent experiments.

over columns (I, II, III) containing increasing amounts of hRPA32C attached to the stationary phase (Figure 14a). After vigorous washing, the eluted fractions were collected and separated on SDS-PAGE. As seen in the figure, eluates from the hRPA32C column contained increasing amounts of Tag-OBD as the amount of RPA32C attached to the beads was increased (lanes 2, 4, 6). To further characterize the interaction, a series of 15N-1H heteronuclear single quantum correlation (HSQC) NMR spectra were acquired for a sample of 15N-enriched Tag-OBD as unlabeled hRPA32C was titrated into the solution. Figure 14b shows binding isotherms for two residues, derived from chemical shift changes induced in the Tag-OBD spectra upon addition of increasing amounts of hRPA32C. The curves were fit to a standard single-site binding equation using the approach described previously (Arunkumar et al. 2003). An average dissociation constant (Kd) of 60 ± 18 μM was obtained from all available data. This binding constant is similar to, but weaker than the Kd of 5-10 μM estimated for the interaction of hRPA32C with peptide fragments from the binding regions of the DNA repair factors XPA and UNG2 (Mer et al. 2000b).

***Structural model of the complex***

The structures of free Tag-OBD (Luo et al. 1996) and hRPA32C (Mer et al. 2000b) have been determined previously. These were used together with NMR chemical shift perturbations to identify the binding sites of the two proteins. Reciprocal titration experiments were carried out using $^{15}$N-enriched RPA32C and $^{15}$N-enriched Tag-OBD with addition of unlabeled partner protein, and these were used to map the binding

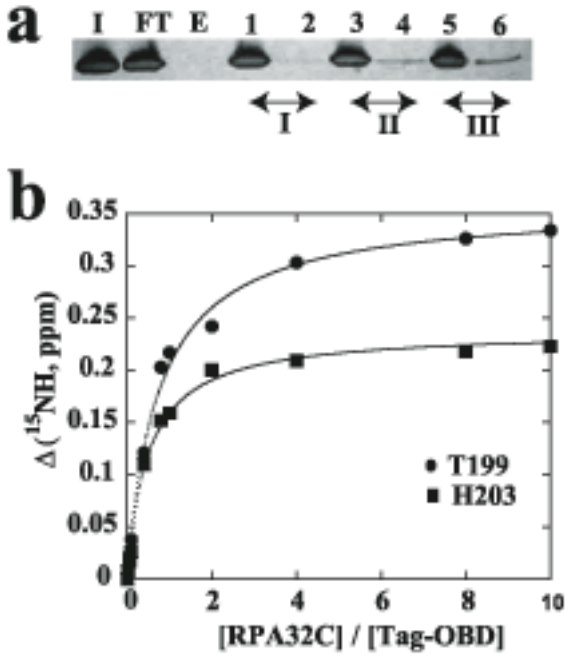


**Figure 14. The interaction of RPA32C with Tag-OBD.** (**a**) Tag-OBD affinity chromatography. Lanes, from left to right: I, column input; FT and E are the flow-through and elution fractions from the control column; 1 and 2 are flow-through and elution fractions from column I; 3 and 4 are flow-through and elution fractions from column II, 5 and 6 are flow-through and elution fractions from column III. (**b**) NMR $^{15}N$ chemical shift titration curves for the binding of RPA32C to $^{15}N$-labeled Tag-OBD. The changes in amide nitrogen chemical shifts of T199 (circles) and H203 (squares) in Tag are plotted against the ratio of Tag-OBD to RPA32C. The line through each curve represents a best fit to the standard single site binding equation.

surface on each molecule (Figure 15 and Figure 16). To determine the structure of the complex, the chemical shift perturbations were used as input to guide a computational docking of the two molecules. The experimental data were sufficient to define a unique relative orientation for the two domains, and multiple refinements converged to an ensemble of conformers with a mean backbone root-mean-square deviation (RMSD) of 0.91 ± 0.17 Å (**Error! Reference source not found.**). The quality of this ensemble is reflected in the backbone dihedral angles, >99% of which are within the allowed regions of the Ramachandran plot. The 20 lowest energy conformers are presented in Figure 17a. The side chains occupying the inter-molecular interface are shown in Figure 17b.

In order to validate and refine the structural model, $^{15}N$-$^{1}H$ residual dipolar couplings ($^{1}D_{N-H}$) were measured by partially aligning the samples in strained polyacrylamide gels. The observed $^{1}D_{N-H}$ values varied from -3 to 5 Hz for RPA32C and from -5 to 7 Hz for Tag-OBD.

The range was sufficiently large for both molecules to enable an accurate assessment versus $^{1}D_{N-H}$ values back-calculated from the structure of the complex derived from the chemical shift perturbation data. There was a good fit between experimental and back-calculated data: the RMSD for the representative structure over all 65 dipolar couplings was only 0.81 Hz (Figure 17c).

***Tag and DNA repair factors bind to the same site on RPA32C***

In the model of the Tag-OBD/RPA32C complex, the binding surface of RPA32C

**Table 2. Structural Statistics of the 20 best RPA32C/Tag-OBD model structures[a]**

| | |
|---|---|
| **No. of residues used in chemical shift perturbation restraints** | |
| From RPA32C | 8 |
| From Tag-OBD | 8 |
| **Ramachandran analysis[b]** | |
| Residues in the favored region (%) | 83 |
| Residues in other allowed regions (%) | 16.5 |
| **Backbone rmsd** (Å) with respect to mean | |
| All backbone | 0.91 ± 0.17 |
| Backbone rmsd (Å) with respect to starting structure | |
| NMR structure of RPA32C | 0.64 ± 0.12 |
| NMR structure of Tag-OBD | 0.48 ± 0.13 |
| **Surface area buried at the inter-molecular interface (Å$^2$)** | 1151 ± 93 |

[a]Structural statistics of the 20 best structures of RPA32C/Tag-OBD complex obtained after flexible docking with HADDOCK followed by refinement in explicit water using ambiguous interaction restraints derived from chemical shift perturbation data. Cluster analysis of the structures was carried out as described in the HADDOCK manual (see Methods section).

[b]Ramachandran analysis was carried out using PROCHECK-NMR (Laskowski et al. 1996).

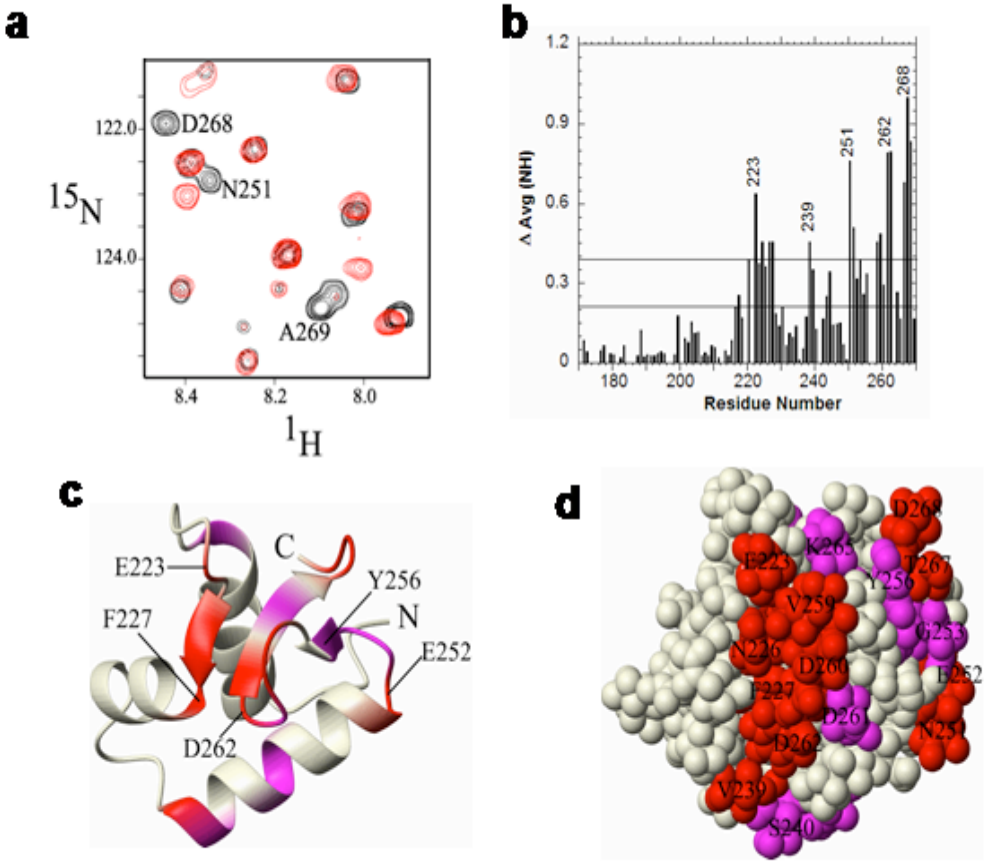


**Figure 15. Mapping the Tag-OBD binding site of RPA32C.** (**a**) $^{15}$N-$^{1}$H HSQC spectra of RPA32C in the absence (black) and presence (red) of 2 molar equivalents of Tag-OBD. (**b**) Bar diagram showing the average chemical shift change (combined amide nitrogen and amide proton) upon addition of 2 molar equivalents of Tag-OBD versus the sequence of RPA32C. The lower bar represents the mean of the data and the upper bar represents the mean plus one standard deviation of the data. (**c,d**) Chemical shift perturbations mapped on the structure of RPA32C shown as a ribbon and a CPK model, respectively. Red coloring indicates those residues that show changes above the mean plus one standard deviation. Magenta represents changes above the mean, but below the mean plus one standard deviation**.**

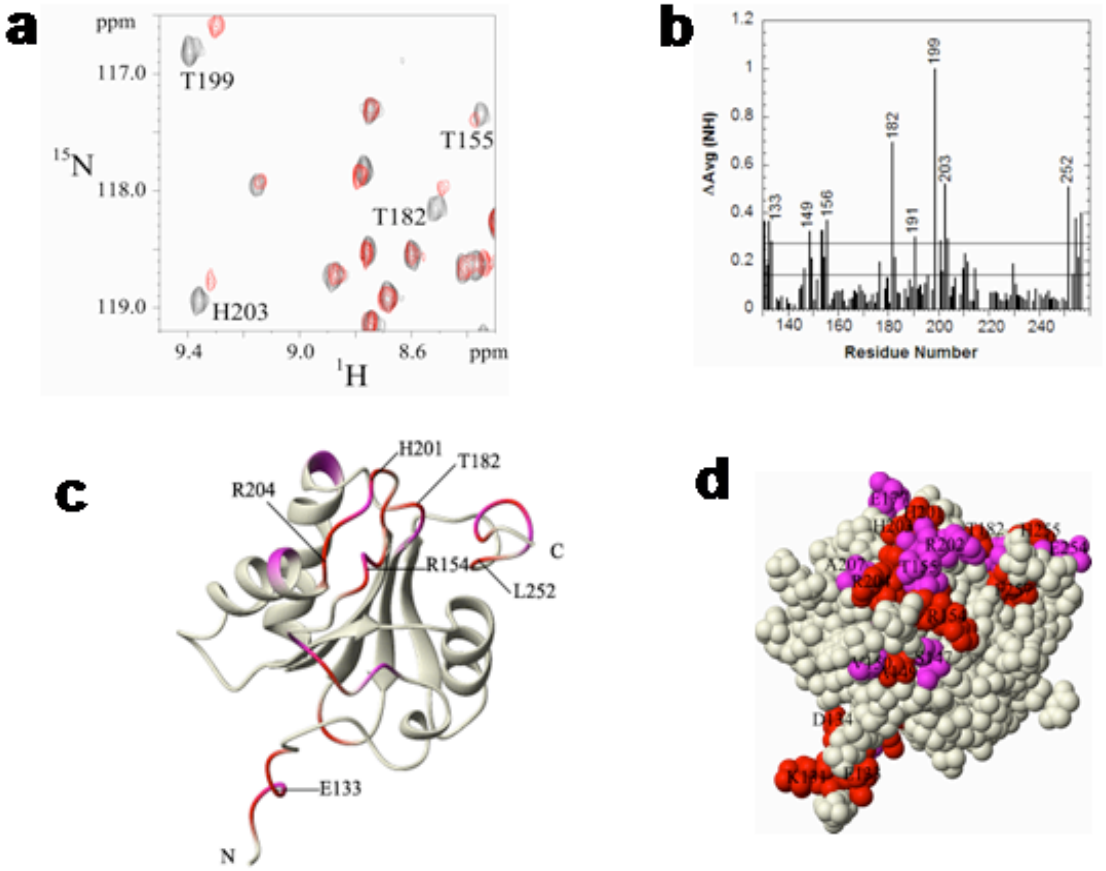


**Figure 16. Mapping the RPA32C binding site of Tag-OBD.** (**a**) $^{15}$N-$^{1}$H HSQC spectra of Tag-OBD in the absence (black) and presence (red) of 2 molar equivalents of RPA32C. (**b**) Bar diagram showing the average chemical shift change (combined amide nitrogen and amide proton) upon addition of 2 molar equivalents of RPA32C *versus* the sequence of Tag-OBD. The lower bar represents the mean and the upper bar represents the mean plus one standard deviation. (**c,d**) Chemical shift perturbations mapped on the structure of Tag-OBD shown as a ribbon and a CPK model, respectively. Red highlights those residues that show changes above the mean plus one standard deviation, and magenta represents changes above the mean, but below the mean plus one standard deviation.

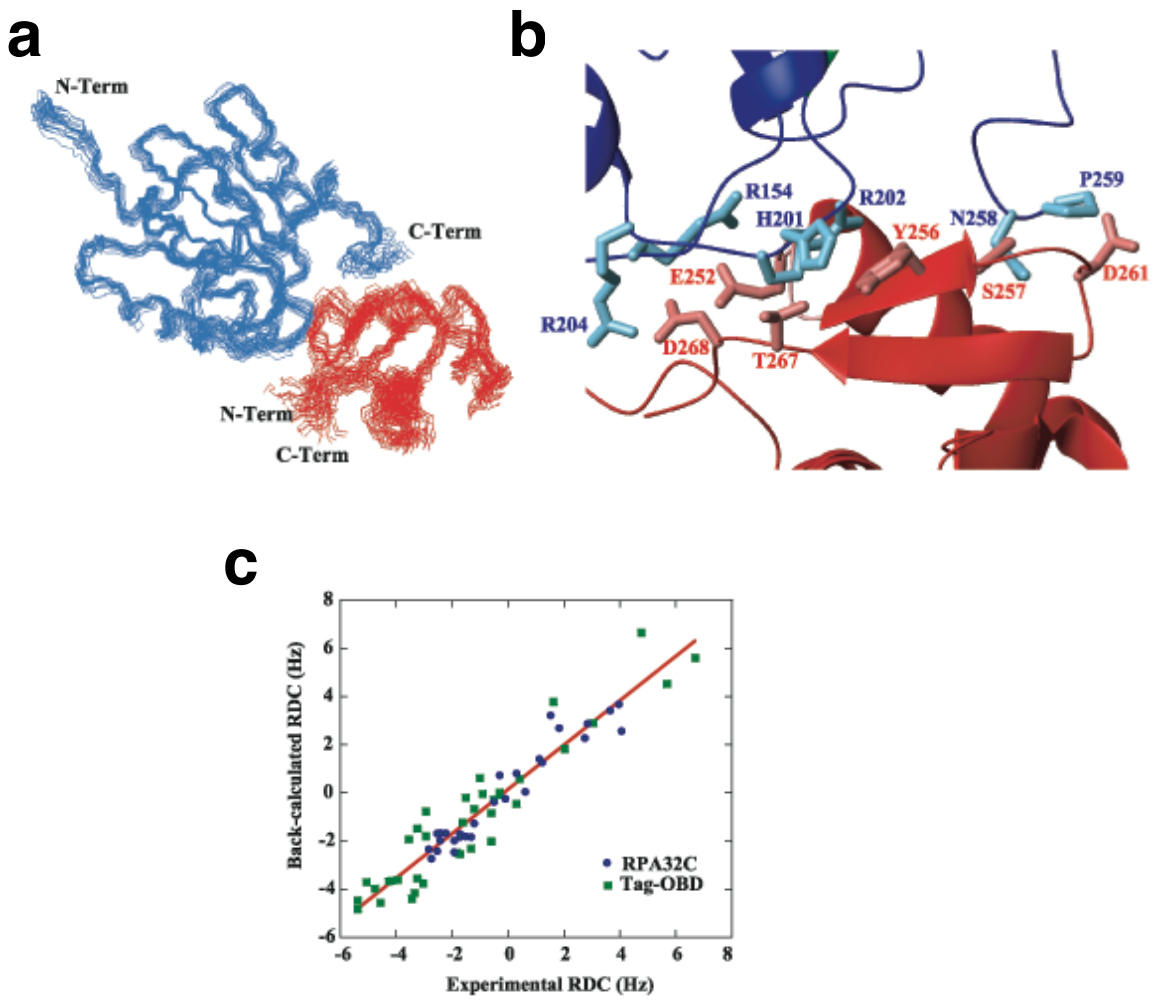


**Figure 17. Docking analysis of Tag-OBD and RPA32C complex.** (**a**) Ensemble of 20 lowest energy conformers of the complex of Tag (blue) and RPA32C (red). (**b**) Side chains in the binding interface of the representative Tag-OBD/RPA32C structure. Tag-OBD is blue and RPA32C is red. (**c**) Correlation between the experimentally measured $^{1}D_{NH}$ dipolar couplings (RPA32C, blue circles; Tag-OBD, green squares) versus the values back-calculated from the representative structure.

includes β-strand II, β-strand III and the loop connecting helix III and β-strand II. There is a striking similarity between the RPA32C complex with Tag-OBD and that with the N-terminal binding region of the base excision repair factor UNG2 (Mer et al. 2000b). Y256 is of particular note because it is a critical residue in the UNG2/RPA32C interface. The participation of Y256 in the Tag complex is clearly evident in $^{13}C$-$^{1}H$ HSQC NMR experiments, which reveal significant perturbations of the aromatic protons of Y256 upon binding to Tag-OBD (Figure 18a). Thus, the similarity between the Tag-OBD and UNG2 complexes appears to extend to the specific details at the binding interface.

Although the structures of RPA32C in these complexes are so similar, the structure of the RPA32C-interacting region of Tag-OBD is distinct from those of the DNA repair factors. In particular, UNG2, XPA and RAD52 all interact with RPA32C through a single helix, whereas the Tag-OBD utilizes a compound surface composed of extended loops (Figure 17b). In addition to R154, R202, R204, N258, and P259, two histidines (H201 and H203) in Tag-OBD are in close contact with RPA32C. There is direct experimental evidence of the presence of the His residues in the binding interface: the resonances of the H201 and H203 side chains in the $^{13}C$-$^{1}H$ HSQC NMR spectrum are significantly perturbed upon addition of RPA32C to a solution of Tag-OBD (Figure 18b). The distinctive character of the Tag-OBD binding site is reflected clearly in the absence of histidine residues in the RPA32C-binding sites of UNG2, XPA and Rad52 (Mer et al. 2000b).

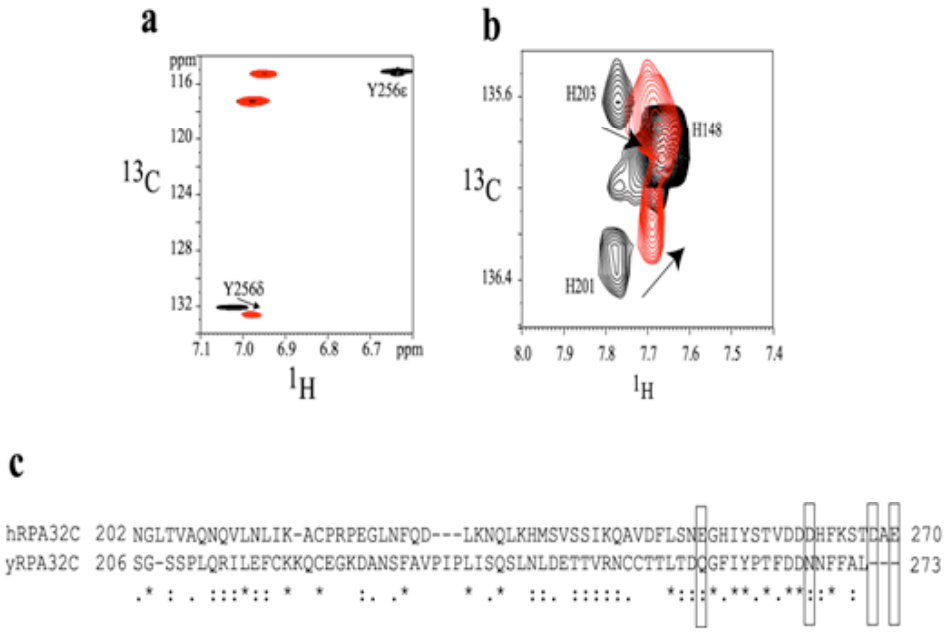


**Figure 18. Side chains at the intermolecular interface.** (**a**) Chemical shift changes in the tyrosine side chain of RPA32C in the aromatic region of the $^{13}C/^{1}H$ HSQC spectrum. (**b**) Chemical shift changes in the histidine side chain of Tag-OBD in the aromatic region of the $^{13}C/^{1}H$ HSQC spectra.

***RPA32C binds to the same site on Tag-OBD as origin DNA***

Detailed analysis of the structure of the complex revealed a significant overlap between the RPA32C-binding surface of Tag-OBD and the previously determined binding site for origin DNA. Three 5-residue regions of the Tag-OBD (F151-T155), (F183-H187) and (H203-A207) have been shown to be essential for origin DNA-specific recognition (Simmons et al. 1990b; Wun-Kim et al. 1993; Luo et al. 1996; Bradshaw et al. 2004). The first indication of similarity between the RPA32C and origin DNA binding sites was from the analysis of chemical shift perturbations, which showed that residues in these three regions of Tag-OBD shift upon addition of RPA32C. Inspection of the model of the Tag-OBD/RPA32C complex reveals that the proposed binding site for RPA32C extends over the top of the deep DNA binding site.

To further confirm the overlap of the Tag-OBD and origin DNA binding sites, a competitive binding experiment was performed on the complex of $^{15}$N-enriched RPA32C and unlabeled Tag-OBD. A duplex DNA oligomer containing the SV40 penta-nucleotide origin sequence recognized by Tag, d(GCAGAGGCCGA)·d(TCGGCCTCTGC), was titrated into this solution to see if the DNA would compete RPA32C off of the OBD. As can be seen by comparing Figure 19a and Figure 19b, the RPA32C signals revert back to the position of free RPA32C upon addition of a stoichiometric amount of DNA. This experiment also shows that origin DNA binds more tightly to Tag than RPA32C, consistent with the reported $K_d$ values for origin DNA (Titolo et al. 2003; Bradshaw et al. 2004) and that noted above for RPA32C.

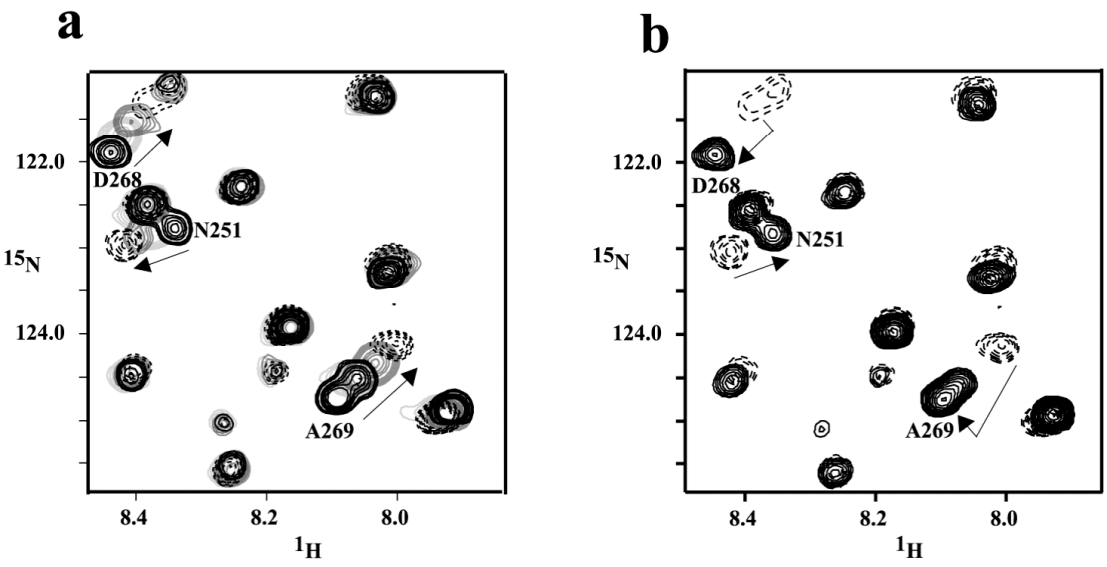


**Figure 19. Effects of DNA binding and mutations on the interaction between RPA32C and Tag-OBD.** Comparison of the binding of Tag-OBD to RPA32C in the absence (**a**) and presence (**b**) of origin DNA. Unlabeled Tag-OBD was titrated into a 100 mM solution of $^{15}$N-enriched RPA32C and a series of $^{15}$N,$^{1}$H HSQC NMR spectra were acquired. An overlay of a small region from these spectra is shown in panel a. A stoichiometric amount of origin DNA duplex was then titrated into the solution and an additional spectrum was acquired, as shown in panel b. Arrows are drawn to facilitate following the change in the location of the NMR signal.

***Mutations in the binding interface inhibit interaction of Tag-OBD and RPA32C***

In order to test the importance of the interaction between the RPA32C domain and the Tag-OBD in SV40 replication, point mutations in RPA32C and Tag-OBD were designed based on the structure of the complex. Inspection of the surfaces of RPA32C and Tag-OBD reveals a significant electrostatic complementarity in their binding surfaces (Figure 20). The RPA32C surface has an acidic character, contributed primarily by E252, D261, D262 and D268. Tag-OBD has three arginine residues (R154, R202, R204) contributing to a complementary basic surface. Salt bridges are found in the binding interface, including the R154 and R204 guanidino groups of Tag-OBD with the carboxylate groups of E252 and D268 of RPA32C, respectively (Figure 17b). The strong electrostatic component of the interaction was confirmed by a salt titration, which revealed that the complex was completely dissociated in 250 mM NaCl (Arunkumar and Chazin, personal communication). Consequently, the design of mutations was based on perturbing electrostatic interactions.

A further test of the proposed importance of electrostatic complementarity between Tag-OBD and hRPA32C involved examining the interaction of Tag-OBD with yRPA32C, which lacks several acidic residues in the hRPA32C binding site for Tag-OBD (Figure 21). The effects of mutations were first assayed by biophysical methods to verify the stability, structural integrity, and binding properties of the mutant RPA32C proteins. Characterization of alanine substitutions of RPA32C residues E252, Y256 and D268 showed that each mutant retained the structure of the wild-type protein, but the effect on affinity for Tag-OBD was only very modest. We reasoned that charge

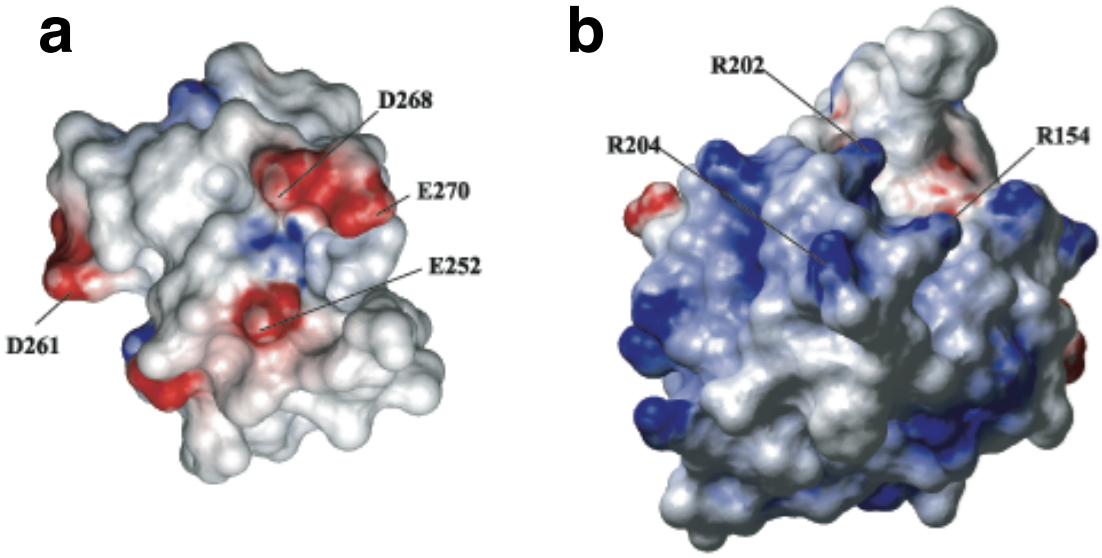


**Figure 20. Electrostatic surfaces of the two molecules in the Tag-OBD/RPA32C complex.** Red and blue colors correspond to negative and positive charge, respectively. Key residues in the binding interface are labeled.

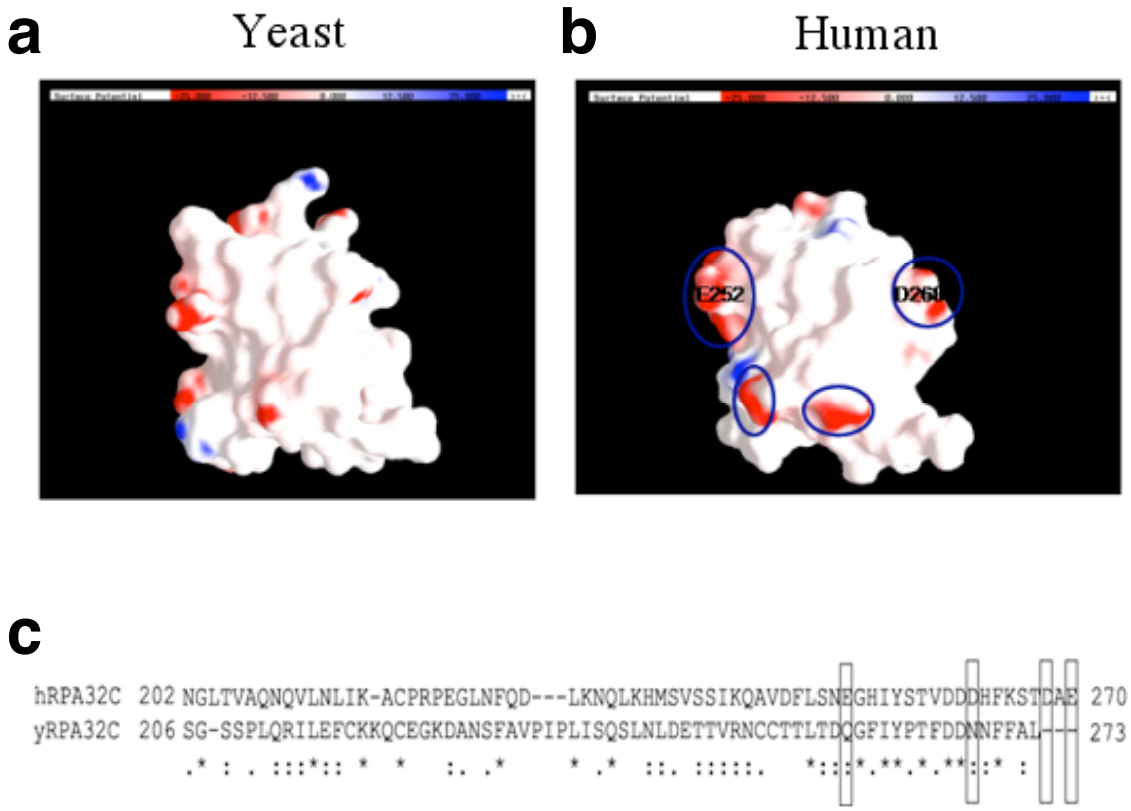


**Figure 21. Comparative analysis of human and yeast RPA32C.** Electrostatic surface representation of human (**a**) and yeast (**b**) RPA32C, generated using hRPA32C coordinates (PDB accession no: 1DPU). Negatively charged residues are red, positively charged—blue. The molecules are in the same orientation. Blue ovals mark the negatively charged patches on human RPA32C surface. Approximate location of the residues used to create charge-reversal point mutants investigated in this study is labeled accordingly. (**c**) Comparison of the primary sequences of the RPA32C domain from human and yeast. Residues that are important for the interaction with Tag-OBD are shown in boxes.

neutralization was insufficient because electrostatic interactions are long-range and not highly directional, so the overall effect could be dispersed through the binding interface. A much more drastic effect was anticipated for charge reversal mutants that place an opposite charge in RPA32C's acidic electrostatic field, and indeed, both E252R and D268R exhibited a more significant effect on Tag-OBD binding. As shown in Figure 22, the binding curves obtained from NMR for E252R and D268R demonstrate 5- to 10-fold weaker binding compared to the wild-type RPA32C ($K_d$ ~500 μM versus 60 μM). Titration of yRPA32C into Tag-OBD revealed a substantially lower affinity than even the most perturbing of the hRPA32C charge reversal mutations (Figure 22). Indeed, binding was so weak that the $K_d$ could not be determined, consistent with the considerably lower negative charge of the Tag-OBD binding surface (Figure 21). RPA32C results were confirmed by similar charge neutralization and charge reversal mutations in Tag-OBD: a modest reduction in affinity for hRPA32C was observed for R154A but a much stronger effect for R154E (Figure 23).

***RPA32C is required for initiation of SV40 DNA replication***

To further assess the functional importance of the proposed Tag-OBD interaction with hRPA32C, hRPA heterotrimers with mutations in RPA32C were tested in SV40 DNA replication assays using the monopolymerase assay (Matsumoto et al. 1990) (Figure 24). Human RPA, used as a positive control in all assays, supported initiation (e.g. Figure 24a, lanes 1, 2). Yeast RPA is unable to support SV40 replication (Melendy and Stillman 1993).

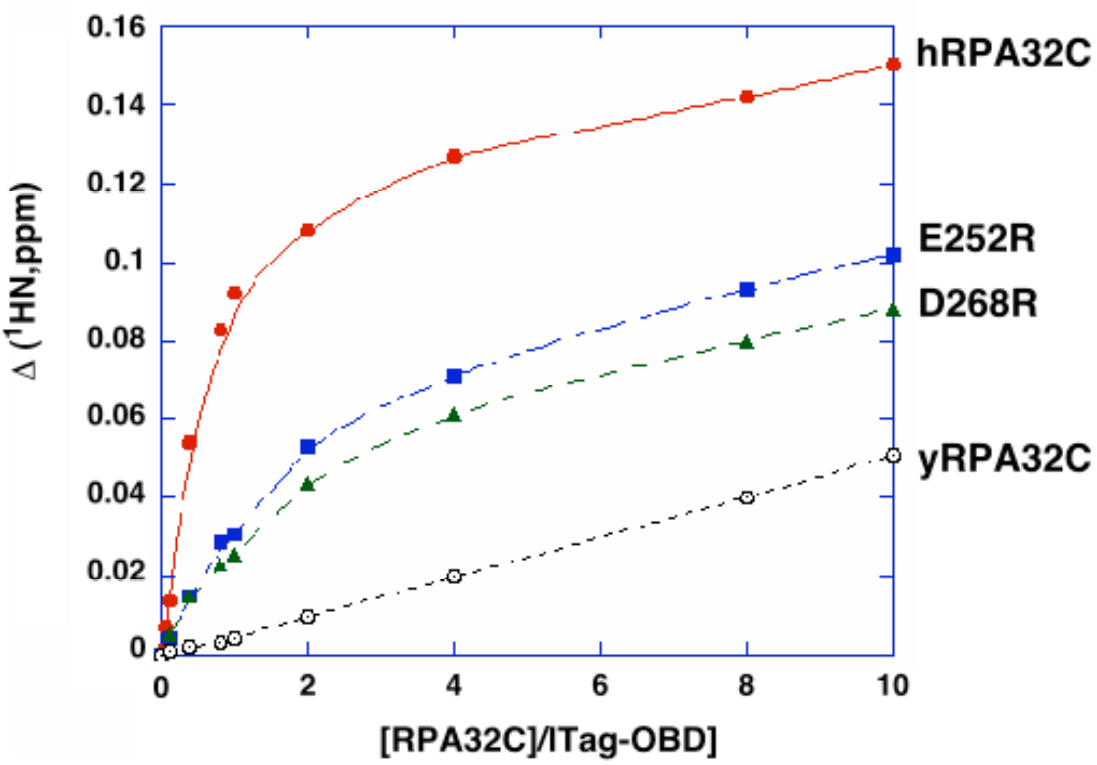


**Figure 22. NMR $^{1}$H chemical shift titration curves for the binding of wild type and point mutants of RPA32C to $^{15}$N-labeled Tag-OBD**. Wild type (squares), E252R (triangles), E268R (filled circles) and yeast (open circles) RPA32C are also labeled to the right. The changes in amide proton chemical shifts of T199 are plotted against the ratio of Tag-OBD to RPA32C. The line through each curve represents a best fit to the standard single site binding equation.

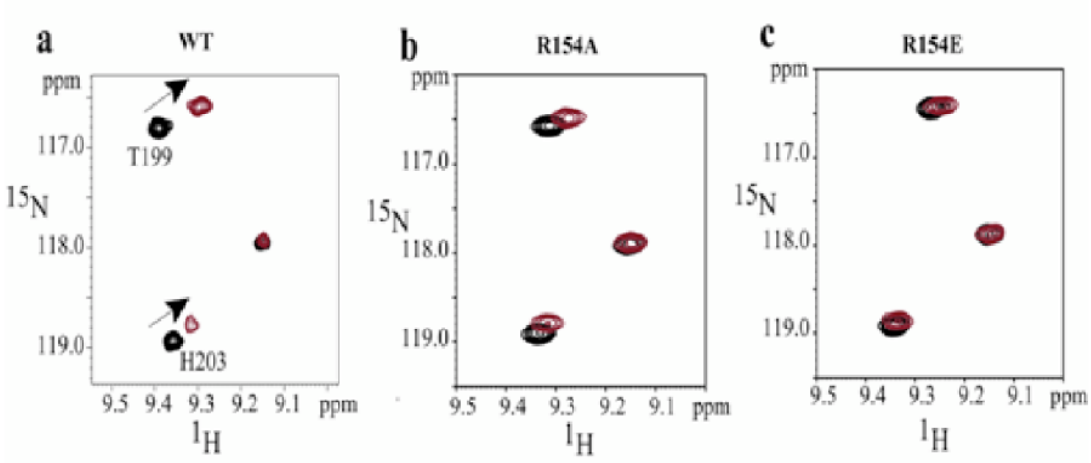


**Figure 23. NMR Chemical shift analysis of the interaction of wild type and mutant Tag-OBD with RPA32C.** (**a**) Expanded region of the $^{15}N$, $^{1}H$ HSQC NMR spectra of wild-type Tag-OBD in free (black) and in complex with 4 molar excess of RPA32C (red). (**b**) Expanded region of the $^{15}N$, $^{1}H$ HSQC spectra of R154A mutant in free (black) and in complex with 4 molar excess of RPA32C (red). (**c**) Expanded region of the $^{15}N$, $^{1}H$ HSQC spectra of R154E mutant in free (black) and in complex with 4 molar excess of RPA32C (red).

A human-yeast chimera hRPAy32C containing the winged-helix-loop-helix domain from yeast RPA in place of the human domain retained ssDNA binding activity and its RPA70 subunit was active in binding to Tag (Figure 12). However, the initiation activity of hRPAy32C was diminished by an order of magnitude relative to that of hRPA in the same experiment (Figure 24a, compare lanes 1-4 and 5-8), correlating with the weak interaction of yRPA32C with Tag-OBD observed by NMR. Initiation activity increased dramatically in proportion to the amount of hRPA present in the reaction, while the corresponding amounts of hRPAy32C did not stimulate replication (Figure 12c). The E252R mutation in RPA32C caused a modest reduction (~20%) in activity relative to wild type hRPA, whereas the D268R mutation substantially impaired initiation activity (~50%) (Figure 24b, c; compare lanes 1-4 and 5-8; Figure 26a-d), consistent with reduced binding of Tag-OBD to the corresponding RPA32C mutants. The results demonstrate that hRPA32C serves an important function in initiation of SV40 DNA replication, and provide strong support for the structural model of the Tag-OBD/RPA32C complex (Figure 17).

***RPA32C interaction with Tag promotes primer synthesis***

To test if the Tag-RPA32C interaction is also needed for primer synthesis at a later step after origin DNA unwinding, ssDNA pre-saturated with hRPA, hRPAy32C chimera, or a point mutant was used as the template for priming and elongation (Figure 25a-c). In the presence of hRPA, Tag stimulated primer synthesis and extension into labeled DNA products (lanes 1-3), while little or no product was detected in the presence

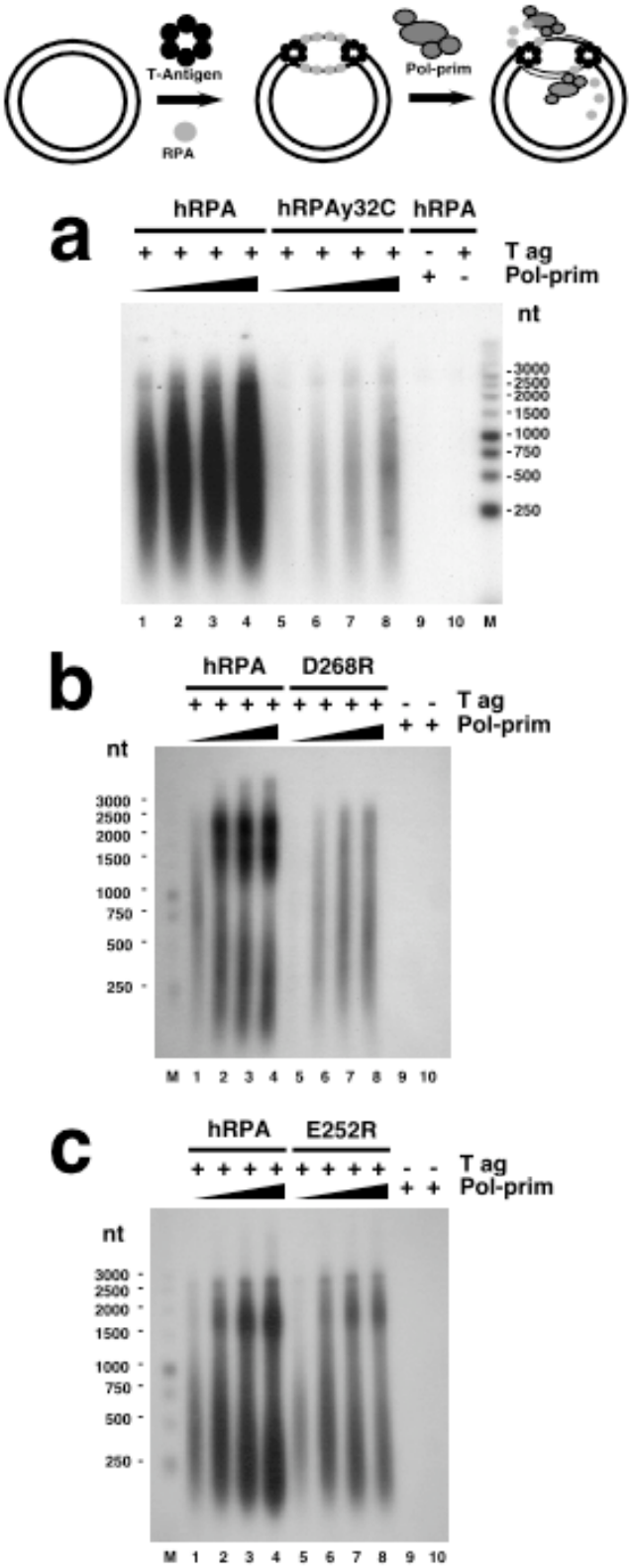


**Figure 24. Mutations in RPA32C that weaken the interaction with Tag are defective in initiation of SV40 DNA replication.** (**a-c**) Initiation of replication was tested in monopolymerase reactions containing 200 ng of the indicated RPA and 100 to 400 ng of pol-prim as indicated. Control reactions contained hRPA but lacked either pol-prim or Tag as indicated (-). The products were resolved by alkaline agarose gel electrophoresis and visualized by autoradiography. DNA size markers are indicated (M).

of any of the mutant RPAs (lanes 6-8). Abundant products were detected in the positive controls without RPA (lanes 12), and little or no products were observed in the absence of pol-prim (lanes 4, 9, 11) or Tag (lanes 5, 10). Quantification of primer synthesis and extension is shown in Figure 24e-h. We conclude that the interaction of Tag with hRPA32C, independent of origin DNA unwinding, is required for its ability to stimulate primer synthesis and elongation on RPA-coated ssDNA.

Previous studies (Bullock 1997), as well as the results presented in Figure 9, suggest that the Tag interaction with RPA is crucial for primer synthesis in initiation and in Okazaki fragment synthesis. To ask if primer synthesis requires the Tag-RPA32C interaction, we first measured the ability of Tag to stimulate the synthesis of radiolabeled RNA primers (8-10 nt) in the SV40 monopolymerase reaction (Matsumoto et al. 1990) in the presenceof wild type or mutant hRPA (Figure 27a). Tag stimulated primer synthesis in the presence of hRPA (lanes 3-5) but not chimeric RPA (lanes 6-8). Similar results were obtained with the E252R and D268R mutants (Figure 27c and d). Control reactions in the absence of Tag or pol-prim (lanes 1-2) yielded no primers. Quantifications of gels were done by phosphorimager analysis and presented in panels d-f. Consistent with SV40 monopolymerase results, the E25R mutant exhibited ~70% of wild type activity, while the D268R mutant retained less than 55% wild type activity (Figure 27g). The results indicate that Tag interaction with hRPA32C promotes priming during initiation.

Additional insights into primer formation were gained by conducting the primer synthesis assay on ssDNA. The reaction was set up in a similar way as described in

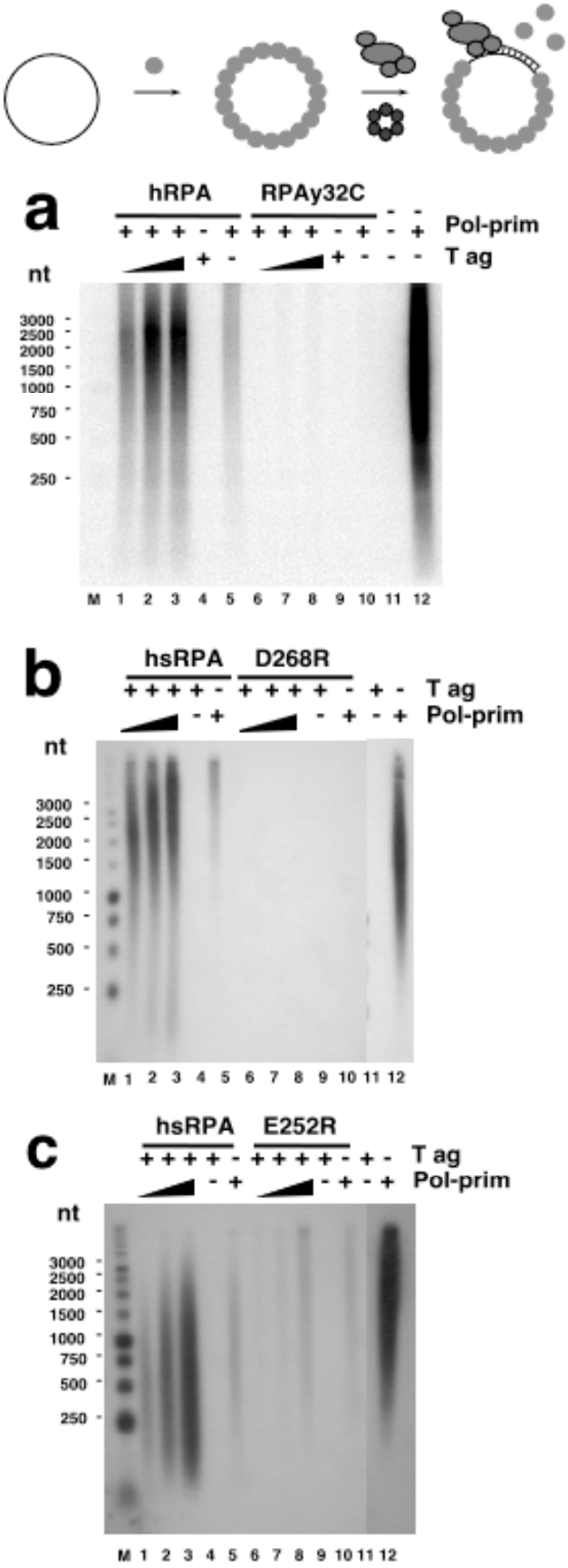


**Figure 25. RPA32C is needed for primosome activity.** (**a-c**) Primer synthesis and extension was assayed on 100 ng M13 ssDNA pre-coated with 600 ng of hRPA or mutant RPA as indicated. Reactions contained 250 ng of pol-prim and 250 to 750 ng of Tag as indicated. Control reactions lacked RPA, pol-prim, or Tag as indicated (-). Radiolabeled DNA products were resolved by alkaline agarose gel electrophoresis and visualized by autoradiography. M, DNA size markers as indicated.

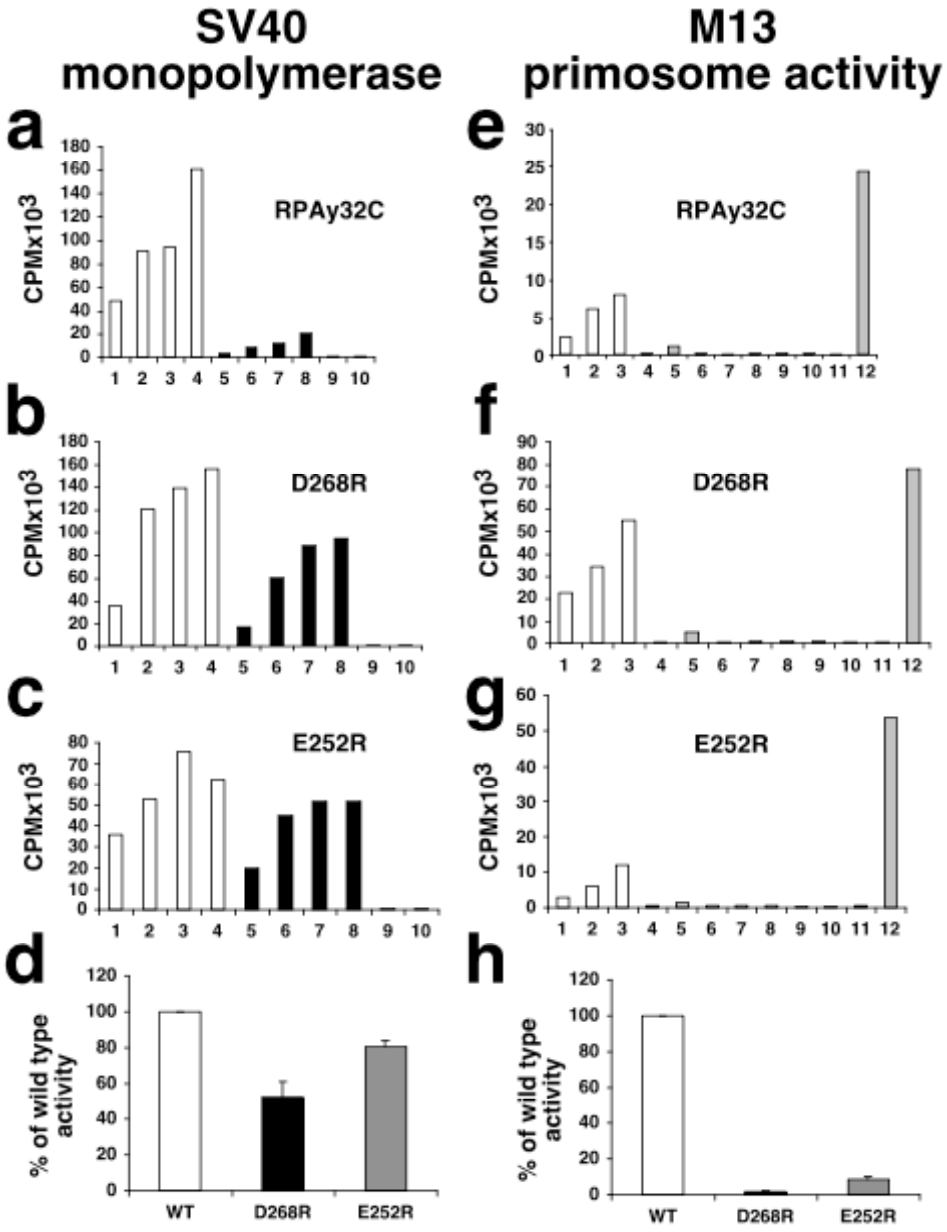


**Figure 26. Quantitative comparison of wild type and mutant RPA in replication assays.** (**a-c**) Quantification of typical SV40 monopolymerase assays as in Figure 24 (cpm, counts per minute determined by scintillation counting). White bars, wild type hRPA; black bars, mutant activity; gray bars, controls. (d) Mean results of at least two independent monopolymerase experiments showing mutant activity as a percentage of the wild type activity in the same experiment. Brackets represent standard error. (**e-g**) Quantification of typical primosome assays as in Figure 25. White, wild type; black, mutant; gray, controls (**h**) Mean results of at least two experiments showing primosome activity with mutant RPA as a percentage of the wild type activity in the same experiment.

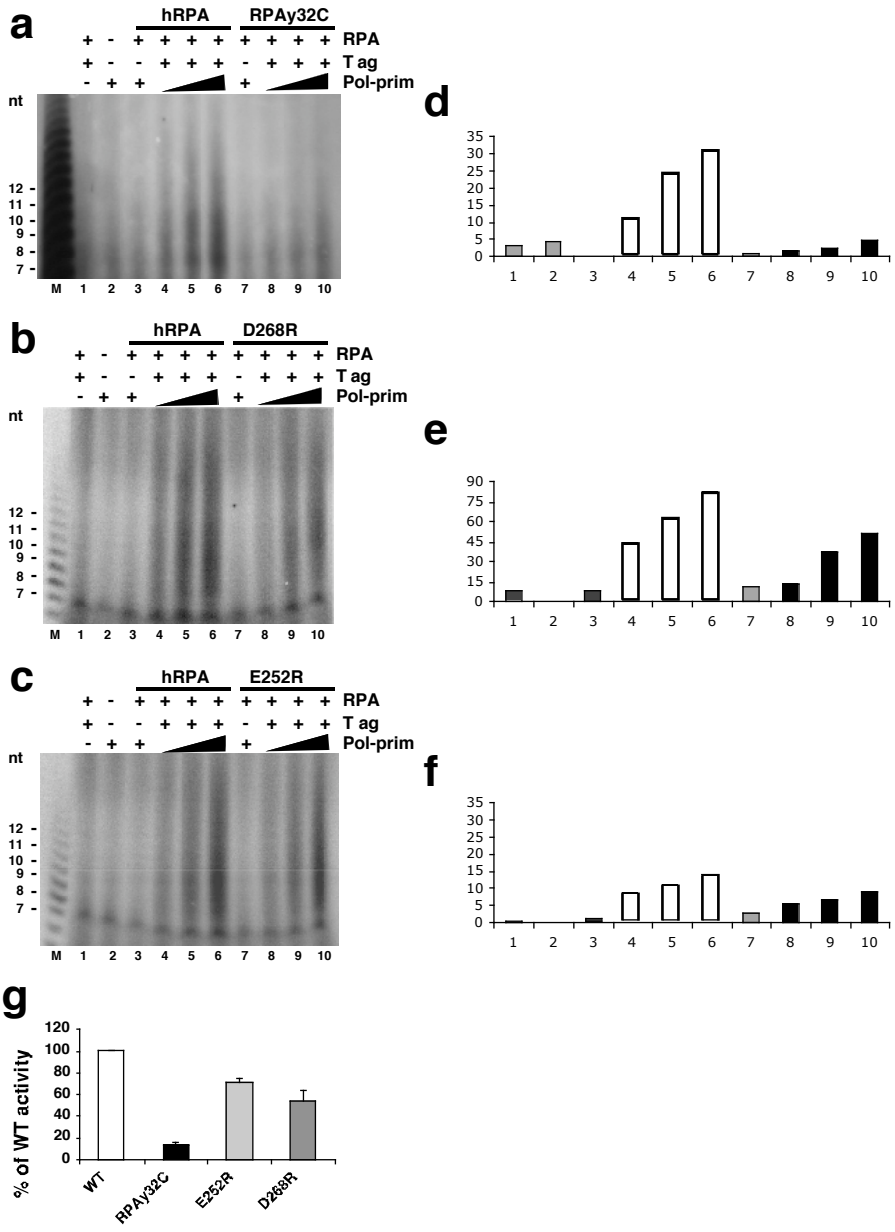


**Figure 27. RPA32C mutants are defective in primer synthesis.** (**a-c**) SV40 monopolymerase reactions containing radiolabeled CTP were carried out in the presence of 200 ng of the indicated RPAs, 250 ng of pol-prim, and 250 to 750 ng of Tag as indicated. Control reactions lacking Tag, RPA, or pol-prim are indicated (-). Radiolabeled RNA products were resolved by electrophoresis on a polyacrylamide gel containing 20% urea and visualized by autoradiography. M, radiolabeled oligonucleotide size marker $dT_{4\text{-}22}$. (**d-f**) Quantification of the reactions using Phosphorimager analysis of the corresponding reactions on the left. (**g**) Primer synthesis in the monopolymerase reaction was quantified by densitometry using IP Lab Gel™ software and expressed as a percentage of wild type activity. At least two reactions were used for quantification of each mutant. Brackets represent standard error.

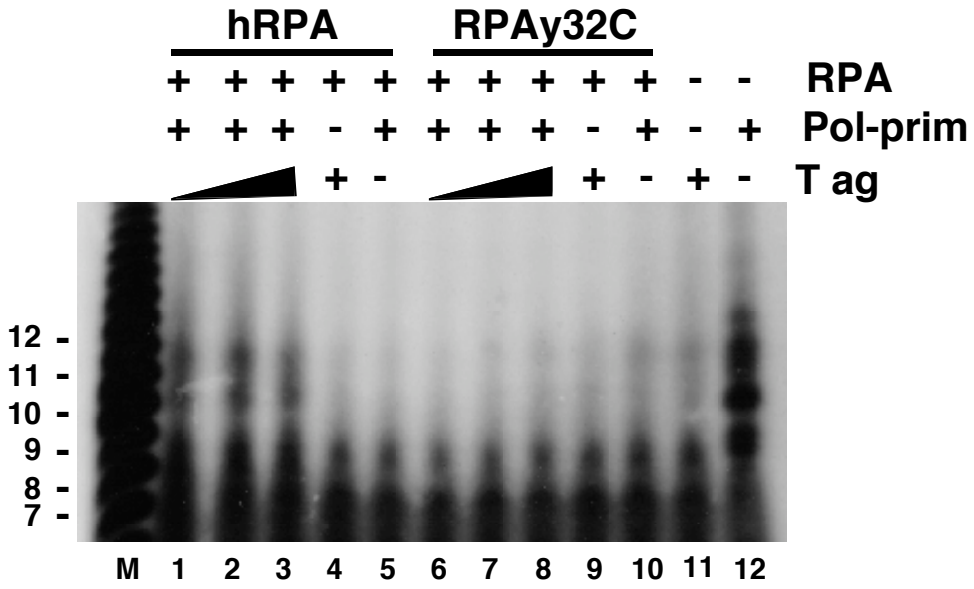


**Figure 28. hRPAy32C mutant does not support Tag-mediated primer synthesis on RPA-coated M13 ssDNA.** Reaction was conducted as in Figure 25 except CTP was the radiolabeled nucleotide, and ATP regeneration system and dNTPs were omitted. The reaction products were processed as in Figure 27.

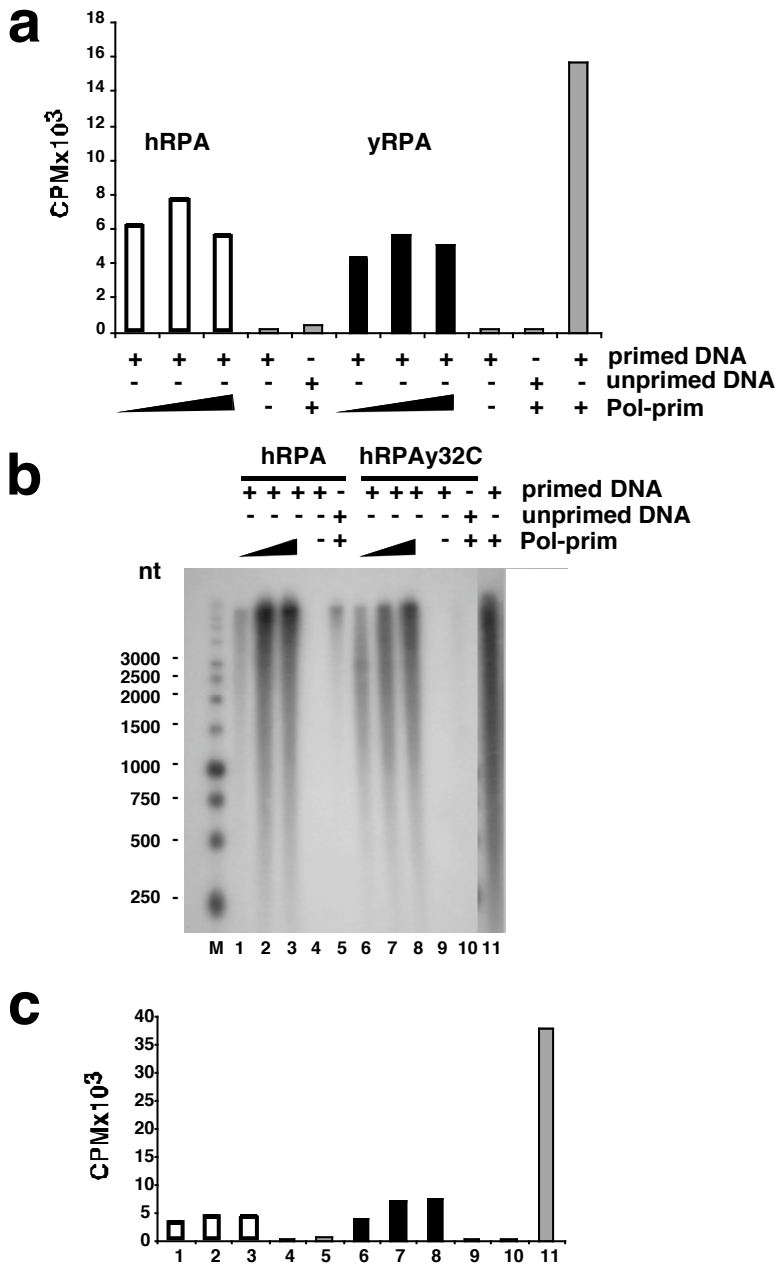


**Figure 29. RPA32 C-terminus is not necessary for primer extension.** (**a**) Singly primed ssDNA (100 ng) pre-coated with 1000 ng of the indicated human or yeast RPA was incubated with purified pol-prim (100, 150, and 250 ng) as indicated (+). Negative control reactions were performed without pol-prim (lanes 4, 9) or with unprimed template (lanes 5, 10). Primer extension in the absence of RPA is shown in lane 11. (**b**) This reaction was done similar to the one described for panel a, but using wt or hRPA32C proteins. (**c**) Quantification of the reaction in panel b as assessed by scintillation counting.

Figure 27, except RPA-saturated M13 ssDNA was used as a template for the reaction (Figure 28). As expected, reactions containing wt RPA yielded abundant primer products (lanes 1-3), as well as positive control lacking RPA (lane 12), while reactions with HRPA32C chimera produced no product (lanes 6-8). Negative control reactions without pol-prim (lanes 4, 9, and 11) or Tag (lanes 5, 10) contained no product. Single point mutants were not tested in this assay,

The data above do not distinguish whether hRPA32C is required only for primer synthesis in the presence of Tag and pol-prim, or also for primer extension, which requires only pol-prim (Bullock 1997; Yuzhakov et al. 1999b). This question was addressed by examining the activity of pol-prim on a pre-primed ssDNA template saturated with either the yeast RPA, chimera hRPAy32C or hRPA (Figure 29a, b). The primer elongation activity in the presence of human, yeast or chimeric RPA was nearly identical (lanes 1-3, 6-8). Primer extension was not detected in the absence of pol-prim or when unprimed DNA template was coated with the same amount of mutant RPA (lanes 4, 5, 9, 10). Primer elongation in the absence of RPA yielded products of smaller size (lane 11), consistent with previous evidence that RPA enhances the processivity of DNA synthesis (Kenny et al. 1990; Matsumoto et al. 1990; Dornreiter et al. 1992; Melendy and Stillman 1993). We conclude that all three RPAs are capable of facilitating primer elongation by pol-prim.

**Discussion**

Taken together with the known interaction of Tag with RPA70 (Braun et al. 1997; Wold 1997; Weisshart et al. 1998; Iftode et al. 1999), the new evidence presented here suggests

that each RPA heterotrimer has two binding surfaces for Tag, one in RPA70 and a weaker one in RPA32C. Although the interaction of RPA32C with Tag-OBD is of moderate affinity, characterization of the complex by NMR enabled modeling of the structure at sufficient resolution to identify critical residues involved in the binding interface. The physical interaction of RPA32C with Tag-OBD is species specific. Despite strong homology between yRPA and hRPA, yRPA32C does not bind Tag-OBD or support SV40 DNA replication. Mutational analysis of hRPA32C strongly suggests that hRPA32C interaction with Tag-OBD allows pol-prim to gain access to hRPA-coated ssDNA for primer synthesis. The reduced replication activity of hRPA32C mutants is easily detectable as the amount of RPA is raised (Figure 12), but might not be obvious with lower amounts, providing an explanation for differences with observations reported previously (Lee and Kim 1995; Braun et al. 1997). Other differences include the use of purified pol-prim and the monopolymerase and primosome assays, rather than the RPA-depleted human cell extracts used previously (Lee and Kim 1995; Braun et al. 1997). Since RPA is highly abundant *in vivo*, our results suggest that protein interactions of RPA32C with Tag are physiologically relevant. Interestingly, a conditional RPA32 mutant of yeast that lacks the RPA32C domain displays slow progression through S phase, synthetic lethality with a conditional pol-prim mutant at permissive temperature, and is nonviable at non-permissive temperature (Santocanale et al. 1995). These phenotypes imply that one or more steps in chromosomal replication may also depend on RPA32C interaction with protein partners.

RPA32C uses a common binding site to interact with Tag-OBD and DNA repair factors (Mer et al. 2000b). Like Tag, XPA and Rad52 have an additional binding site in

RPA70 (Jackson et al. 2002; Daughdrill et al. 2003), although the relative importance of these contact points in DNA repair is not known. Notably, the RPA32C deletion mutant of yeast displays a mutator and hyper-recombination phenotype, which does suggest a role for RPA32C in DNA repair (Santocanale et al. 1995). One of the common functions of RPA in these different DNA processing pathways lies in its ability to facilitate the exchange of proteins on ssDNA ("hand-off") (Kowalczykowski 2000; Mer et al. 2000b; Stauffer and Chazin 2004) as the pathway proceeds. The promiscuity of RPA32C in binding DNA processing proteins, while maintaining modest affinity for its binding partner, suggests that it serves as a facilitator in the hand-off mechanism. Characterization of structural mechanisms such as hand-off presents a significant challenge, but one which must be overcome in order to better understand fundamental DNA processing events such as replication.

***How does hRPA32C promote T antigen-mediated primer synthesis?***

Primer synthesis but not primer elongation on RPA-saturated ssDNA requires Tag (Matsumoto et al. 1990; Collins and Kelly 1991; Melendy and Stillman 1993; Yuzhakov et al. 1999b). The ability of Tag to mediate priming by pol-prim correlates with its ability to interact physically with the RPA bound to the template, strongly suggesting that physical interactions of Tag with RPA facilitate priming (Melendy and Stillman 1993; Weisshart et al. 1998). The data presented in this report and previously (Lee and Kim 1995) point to a functional role for RPA32C in Tag-mediated priming.

How might the physical interaction of Tag with RPA32C facilitate primer synthesis? We postulate that Tag interacts with RPA to facilitate its partial dissociation

from ssDNA, thereby creating a short region of ssDNA accessible for primer synthesis (Figure 30). Based on all available evidence (Wold 1997; de Laat et al. 1998; Iftode et al., 1999; Yuzhakov et al. 1999b; Iftode and Borowiec 2000; Bastin-Shanower and Brill 2001; Ott et al. 2002a; Arunkumar et al. 2003; Bochkarev and Bochkareva 2004), we propose that a dual interaction of Tag with RPA32C and RPA70 allows it to remodel the structure of ssDNA-bound RPA, transiently shifting it from the high affinity, extended binding mode to a weaker, more compact binding mode (Figure 30a, b). RPA binds to ssDNA with the high affinity DNA binding domains A and B at the 5' end of the occluded ssDNA, followed by the weaker binding domains C and D at the 3' end. This implies that the 3' ssDNA would be transiently accessible simply due to the lower affinity of RPA domains C and D for ssDNA. Binding of Tag to RPA32C might prolong the time window in which the 3' site is accessible. Since Tag binds to pol-prim through its helicase domain, a single Tag hexamer may bind concurrently to RPA and pol-prim (Fanning and Knippers 1992; Bullock 1997; Weisshart et al. 1998; Simmons 2000; Stenlund 2003). Given that Tag binding to both proteins is essential for priming (Weisshart et al. 1998), we propose that a Tag hexamer transiently associated with both RPA and pol-prim is poised to load pol-prim onto the accessible region of ssDNA (Figure 28b,c). Primase would thereby gain access to the free ssDNA template, permitting primer synthesis and leading to dissociation of a remodeled RPA molecule and Tag. Subsequent primer extension on RPA-ssDNA by pol-prim does not require Tag (Figure 30d). The model proposed in Figure 30 is consistent with our results and a large body of published evidence, but much work remains to assess its validity.

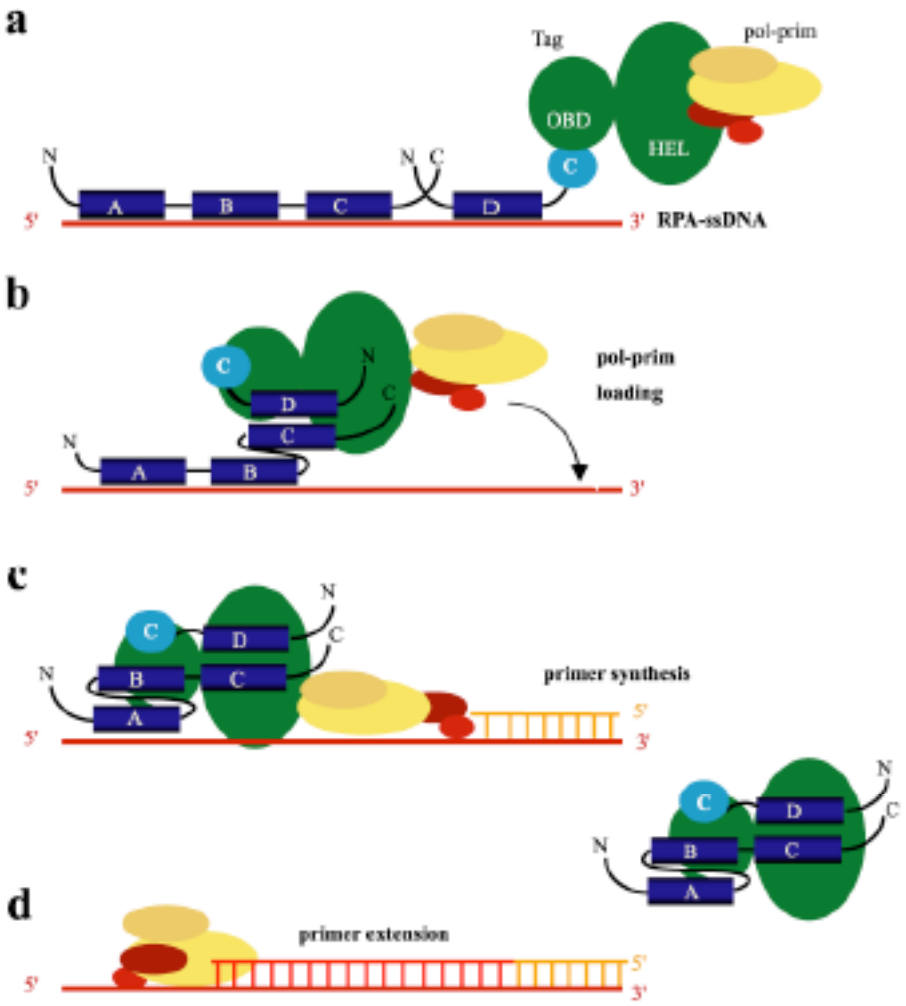


**Figure 30. Model for SV40 primosome activity on RPA-coated ssDNA.** (**a**) RPA (blue) is schematically depicted in the high affinity 28-30 nt binding mode with all four ssDNA binding domains (A-D) bound to ssDNA. RPA14 is omitted for simplicity. The helicase domain (HEL) of a Tag hexamer (green) can associate with a pol-prim heterotetramer (Koradi et al. 1996; Bullock 1997). Antibodies against Tag that specifically inhibit either RPA binding to Tag-OBD or pol-prim binding to the helicase domain prevent primer synthesis (Weisshart et al. 1998). (**b**) We suggest that primosome assembly begins when Tag-OBD associates first with RPA32C and then with RPA70AB, transiently creating a short stretch of unbound ssDNA. (**c**) In concert with this RPA remodeling, pol-prim associated with the Tag hexamer would be poised to access the free ssDNA and begin primer synthesis. (**d**) Primer extension by pol-prim is likely coupled with RPA and Tag dissociation, and followed by the RFC/PCNA-mediated switch to DNA polymerase delta (Huang et al. 1998) (not shown).

In order to better understand the role(s) of RPA32C, it will be necessary to complete the analysis of Tag-RPA interactions. To this end, ongoing studies are focused on mapping the interaction sites of RPA70AB to determine how interactions of both Tag-interacting regions of RPA are coordinated, and regulated through their interactions with DNA and pol-prim, during the initiation of SV40 DNA replication. The increasingly detailed knowledge of the mode of action of modular, multi-functional proteins, such as our studies of the SV40 replisome, are of considerable value because they will develop a deeper understanding of the fundamental molecular mechanisms of DNA processing machinery and the role of RPA in guiding the succession of proteins in each pathway.

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CHAPTER III

# REQUIREMENTS FOR HCDC6 PHOSPHORYLATION IN INITIATION OF MAMMALIAN DNA REPLICATION AND NUCLEAR EXPORT

## Introduction

DNA replication is a complex and tightly regulated process essential for cell cycle progression. Pre-replication complex (pre-RC) formation constitutes the initial step in committing a cell to enter S phase. Several gene products were identified that are required for pre-RC formation. Human Cdc6 (hCdc6) is a key regulator of pre-RC formation. hCdc6 is recruited to chromatin by the origin recognition complex (ORC) as cells transit from mitosis to G1 (for review see (Bell and Dutta 2002; Pelizon 2003; Oehlmann et al. 2004; Machida and Dutta 2005)). Studies in yeast and *Xenopus* indicate that hCdc6 together with Cdt1 loads MCM complex, a putative helicase (Fujita 1999; Nishitani et al. 2000). The current model proposes that Cdc6 is phosphorylated by CDKs, exported from the nucleus or degraded through a ubiquitin-dependent pathway after loading MCM2-7 (Figure 31a). However, sub-cellular localization of hCdc6 in cells synchronized with aphidicolin or hydroxyurea/thymidine suggests that MCM loading by hCdc6 is not sufficient for its nuclear export in human cells. As reported in several studies, human Cdc6 localizes to the nucleus in cells blocked with aphidicolin and to the cytoplasm in cells treated with hydroxyurea/thymidine (Saha et al. 1998; Jiang et al. 1999; Petersen et al. 1999). Aphidicolin blocks replicative polymerases, threreby stalling the replication forks Figure 31b. The drug uncouples the polymerase and primase

activities of the polymerase-α-primase. Thus, the aphidicolin treated polymerase lays primers, but cannot replicate DNA. The aphidicolin blocked pre-replicative complex is fully formed and contains replicative polymerases. According to the current model, hCdc6 should be exported from the nucleus at this point in initiation since the MCM 2-7 complex has already been loaded by hCdc6. However, under these conditions, hCdc6 remains in the nucleus and is presumably chromatin-bound. Hydroxyurea and thymidine elicit a replication block in the cell cycle by inhibiting the action of the ribonucleotide reductase enzyme. Cells entering S phase are able to lay primers and replicate short fragments of DNA when propagated in hydroxyurea/thymidine-containing medium, but replication stops due to ribonucleotide reductase inhibition and the resulting depletion of dNTP substrates (Figure 31b). These data might indicate that replication initiation is required for hCdc6 nuclear export.

Human cells synchronized with pharmaceutical agents that block cells at the G1/S transition demonstrate that hCdc6 is nuclear in G1 and is gradually exported from the nucleus as cells progress into S phase (Saha et al. 1998; Jiang et al. 1999; Petersen et al. 1999). Cyclin dependent kinase phosphorylation of hCdc6 promotes its nuclear export after MCM loading (Herbig et al., 2000). hCdc6 possesses nucleotide binding motifs designated Walker A and Walker B, and Sensor I and Sensor II motifs that distinguish ADP from ATP bound states (Figure 32) (Perkins and Diffley 1998; Liu et al. 2000; Schepers and Diffley 2001). Studies in the *Xenopus* cell-free system indicate that Walker A and Walker B motifs are required for hCdc6's proper function in loading MCMs on chromatin (Frolova et al. 2002). Similar requirements exist in budding yeast (Takahashi et al. 2002) and humans (Herbig et al. 1999). Sequence analysis of Cdc6 revealed

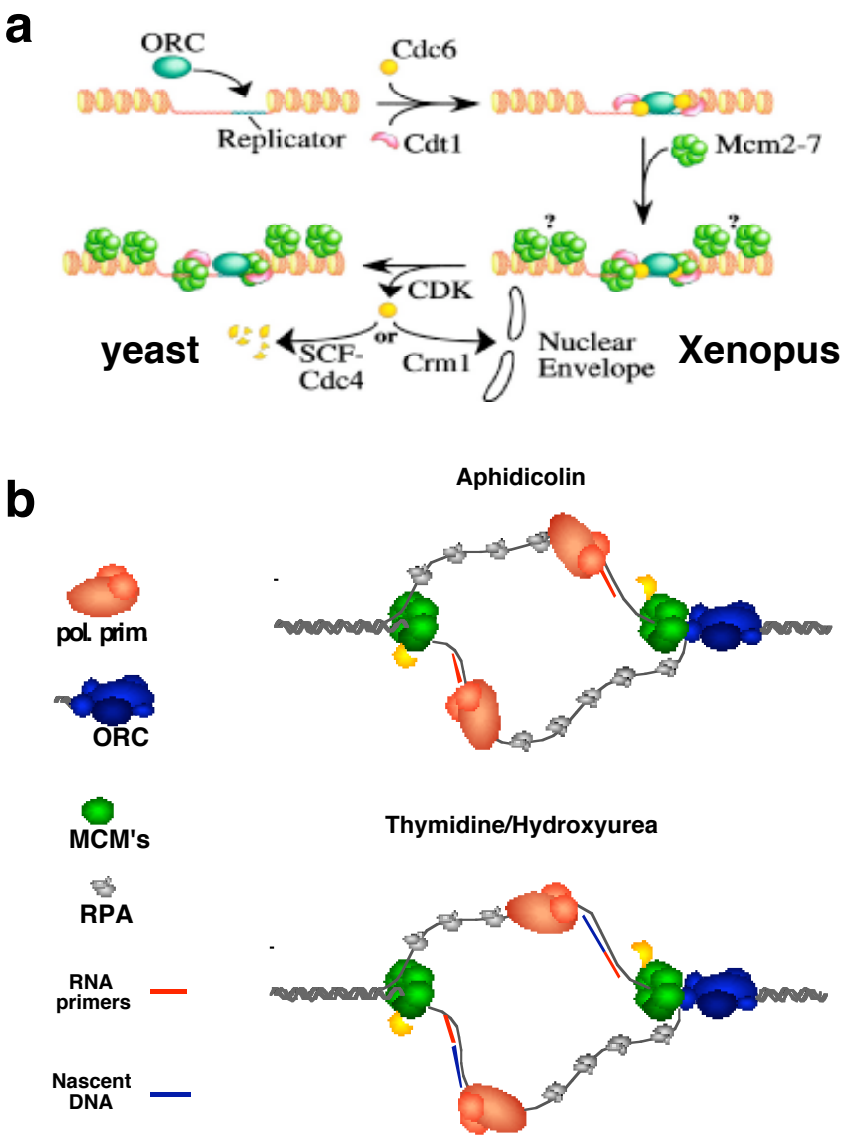


**Figure 31. A current model of the initiation of DNA replication, the role and timing of Cdc6 activity.** (**a**) Current model of initiation of DNA replication, based primarily on the studies in Xenopus (right) and yeast (left). Adapted from Bell and Dutta, Annu. Rev. Biochem., 2002. (**b**) A model demonstrating the specific time during cell cycle when aphidicolin or thymidine/hydroxyurea exert its action.

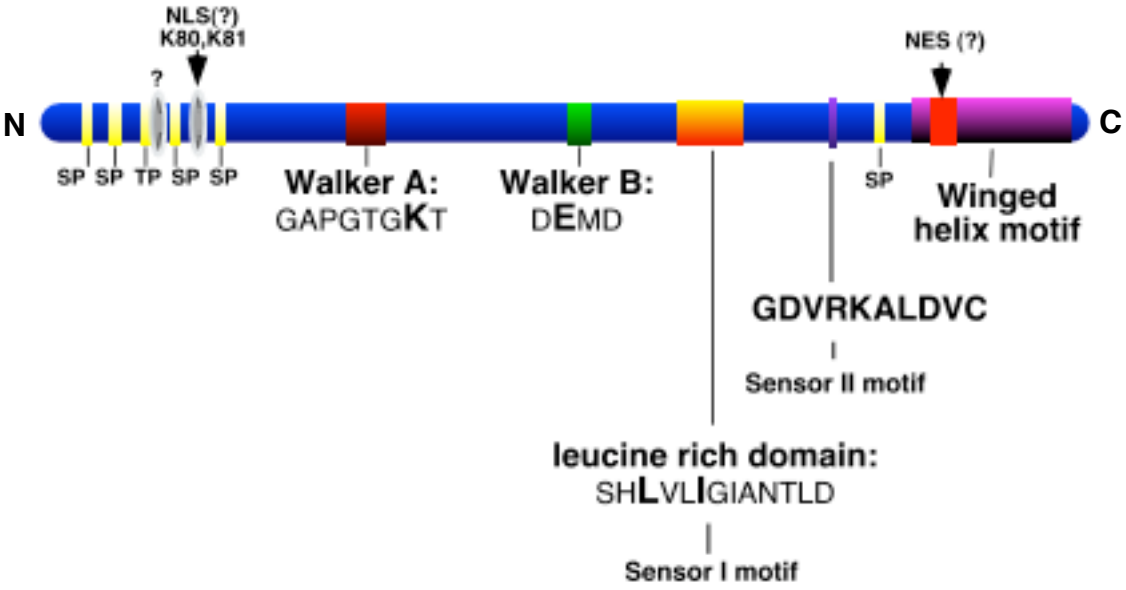


**Figure 32. A model showing known functional motifs of hCdc6.** The mutated residues are in bold. Phosphorylation sites (yellow lines) S45, S54, T67, S74, and S106 are located at the N-terminus; a potential phosphorylation site S419 is also labeled. Approximate position of NLS (grey ovals) and NES (red rectangle) are shown as proposed by Delmolino et al., JBC, 2001.

several nucleotide-interacting domains characteristic of AAA+ family of proteins: Walker A, Walker B, Sensor I, and Sensor II. Genetic (Perkins and Diffley 1998; Schepers and Diffley 2001), structural (Liu et al. 2000), and biochemical (Herbig et al. 1999; Frolova et al. 2002) analyses of these motifs allowed the determination of the role of some of these domains in regulating Cdc6 action. The Walker A motif is necessary to bind ATP, while the Walker B motif is required for Cdc6 to hydrolyze the nucleotide (Herbig et al. 1999; Frolova et al. 2002). The Sensor I motif was speculated to allow the protein to distinguish whether the ADP or ATP form of the nucleotide was bound. Expressed alone, the sensor I motif mutants were shown to be dominant-negative in yeast (Schepers and Diffley 2001) and human cells (Griffith, 1999, Honors thesis). The Sensor II mutations are lethal and the motif's exact function is still enigmatic (Schepers and Diffley 2001).

Preliminary studies suggest that phosphorylation of hCdc6 in human cells is required for replication and that phosphorylation-defective mutants exhibit dominant-negative phenotypes (Jiang et al. 1999; Herbig et al. 2000). In the yeast model, phosphorylation is not required for replication, but phosphorylation induces ubiquitination and proteolysis after initiation of replication (Wolf et al. 1999; Calzada et al. 2000; Jang et al. 2001). Similar findings were reported in the *Xenopus* system, in which phosphorylation of Cdc6 is not essential for replication, but it facilitates the protein's chromatin dissociation after initiation of replication (Coleman et al. 1996; Mendez and Stillman 2000; Pelizon et al. 2000; Gillespie et al. 2001). In these model systems, phosphorylation of Cdc6 prevents re-initiation of replication. However, little is known in human cells about the role of hCdc6 phosphorylation in DNA replication, the

sequence of events leading to DNA replication, and the exact mechanism of Cdc6 export from the nucleus.

To address hCdc6 function in human DNA replication, we utilized GFP-tagged human Cdc6 protein with mutations in phosphorylation and nucleotide interacting domains for sub-cellular localization, replication kinetics, and ability to replicate DNA in synchronized HeLa cells. Our findings suggest that functions of both nucleotide-binding and Sensor domains are required for hCdc6 ability to function and exit the nucleus. Moreover, phosphorylation alone is not sufficient for hCdc6 nuclear export, and hCdc6 export from the nucleus occurs gradually over time in S phase. Our studies also suggest the existence of a secondary mechanism required for hCdc6 nuclear export, possibly involving prolyl bond isomerization by human Pin1 or another prolyl isomerase.

## Materials and methods

### *Cdc6 mutant construction*

Cdc6 mutants were generated according to the QuikChange™ Site-Directed Mutagenesis protocol (Stratagene). Mutant DNA was generated using GST-hCdc6 in pBS KS II + (Herbig et al. 1999) as a template and the following primers: S45D forward 5'-CCAAACCGTAACCTGTGATCCTCGTGTAAAAGCC-3' and reverse 5'-GGCTTTTACACGAGGATCACAGGTTACGGTTTGG-3', S54D forward 5'-GTAAAAGCCCTGCCTCTCGACCCCAGGAAACGTCTGGG-3' and reverse 5'-CCCAGACGTTTCCTGGGGTCGAGAGGCAGGGCTTTTAC-3', T67D forward 5'-

GACAACCTATGCAACGATCCCCATTTACCTCC-3' and reverse 5'-GGAGGTAAATGGGGATCGTTGCATAGGTTGTC-3', S74D forward 5'-CCATTTACCTCCTTGTGATCCACCAAAGCAAGGC-3' and reverse 5'-GCCTTGCTTTGGTGGATCACAAGGAGGTAAATGG-3', S106D forward 5'-CAGCTGACAATTAAGGATCCTAGCAAAAGAG-3' and reverse 5'-CTCTTTTGCTAGGATCCTTAATTGTCAGCTG-3', Walker A forward 5'-CCTGGAACTGGAGCAACTGCCTGC-3' and reverse 5'-GCAGGCAGTTGCTCCAGTTCCAGG-3', Walker B forward 5'-GGTATTGGACGCGATGGATCAACTGG-3' and reverse 5'-CCAGTTGATCCATCGCGTCCAATACC-3'. The mutations were generated using 50 ng template DNA, 125 ng of each primer, and 10 mM dNTP mix in 50 μl reaction volume. Polymerization was performed by PfuTurbo polymerase (Stratagene) in 1X Pfu buffer for 18 cycles in a Perkin Elmer 480 thermal cycler (95°C for 30 sec, 55°C for 1 min, and 68°C for 11 min). GFP fusion constructs were produced by digesting pBluescript KS II+ plasmid containing Cdc6 mutants with BstUI for 1 hr at 60°C. pEGFP-C2 (Clontech) vector was digested with SmaI for 1 hr at 25°C. Both the vector and the insert were gel-purified and ligated overnight at 16°C by T7 ligase (Clontech) in its buffer. DNA plasmids were propagated in HB101 competent cells. Introduction of all mutations was verified by DNA sequencing.

***Cell culture and cell synchronization***

HeLa cells were grown in a monolayer in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Gaithersburg, MD) supplemented with an antibiotic cocktail

(streptomycin, kanamycin, and strepticillin), L-glutamine and 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA) in humidified incubator at 37° C and 5% $CO_2$. Exponentially growing HeLa cells at approximately 70% confluency were blocked in G2/M for 18-20 hrs with 50 ng/ml nocodazole (Sigma). Cells were then harvested by mitotic shake-off and released into G1 by washing two times with fresh media. Released cells were plated on cover glass in 35 mm cell culture dish (Corning Inc.) for microinjection.

***Microinjection***

Cells for microinjection were incubated for 4-6 hr following the release from nocodazole block to allow them re-attach to the matrix. DNA plasmids for microinjection were diluted in 0.2 $\mu$m filtered microinjection buffer (25 mM HEPES-KOH, pH7.5 and 50 mM NaCl) to a final concentration of 100 ng/□l. At least 2 hrs of incubation after injections were allowed for the protein to be expressed before the cells were fixed. All samples for microinjection were centrifuged for 60 min at 14,000X g. Microinjections were accomplished using a microinjector (model 5246; Eppendorf Scientific, Madison, WI) and a manipulator (model 5171; Eppendorf Scientific, Madison, WI). Cells in the Petri dishes for microinjection were mounted and observed on an inverted microscope (model IM35; Zeiss, Oberkochen, Germany). Needles were pulled from borosilicate glass capillaries (Clark Electromedical Instruments, Reading, UK) on an automatic DMZ-Universal Puller (Zeitz Instruments, Augsburg, Germany).

***BrdU incorporation***

In order to monitor DNA replication, media was supplemented with 10 μM bromodeoxyuridine (BrdU; Sigma) two hours before fixation. Cells were fixed as follows: washed 3 times in ice-cold phosphate buffered saline (PBS), incubated 20 min in 3% paraformaldehyde in PBS, washed 3 times with PBS, permeabilized for 20 min with 0.2% Triton-X100, washed 3 times with PBS, and incubated for 45 min in PBS supplemented with 10% FBS. Mouse α-BrdU antibody (Amersham, Arlington Heights, IL) at a 1:10 dilution supplemented with 10 units of Benzonase (Novagen) was incubated with the cells for two hours followed by 3 washes with PBS and 3 washes of 5 min each with PBS. Cells were then incubated with α-mouse Cy-3 conjugated secondary antibody at a dilution 1:100 (Jackson ImmunoResearch Laboratory, Inc.) for one hour, followed by the same washing protocol as for α-BrdU antibody. DNA was stained by 10 min incubation with 2 μg/ml Hoechst dye in PBS, followed by three washes with PBS. Cover glasses with cells were dried overnight and mounted with a ProLong antifade kit (Molecular Probes). Fluorescence was observed using an inverted fluorescent microscope (model Axiovert 135, Carl Zeiss, Oberkochen, Germany). Digital pictures were obtained with an RT Monochrome camera, power supply, and manufacturer's software Spot™ 3.0.5 (Diagnostic Instruments, Inc, Sterling Heights, MI).

***Expression and purification of recombinant hCdc6 protein***

Wild type and mutant GST-hCdc6 proteins were expressed from baculovirus vectors in Hi5 insect cells and purified according to the protocol previously described by Herbig et al., (1999).

***Partial tryptic digest of GST-hCdc6 protein***

Freshly purified GST-hCdc6 protein bound to glutathione-agarose resin (Sigma) was washed 2X with PBS, equilibrated by washing 2X with buffer D (20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, and 8 mM $MgCl_2$), and digested for 10 min at 37°C in a total reaction volume of 20 μl. The reactions contained 0.5 μg GST-hCdc6 protein and either no nucleotide or 2 mM ATP, ATPγS, ADP, or UTP in buffer D. The amount of trypsin necessary for digestion was empirically determined by titration in each case. The reactions were stopped by addition of SDS sample buffer and boiled for 5 min at 95°C. The proteolytic products were resolved on 10% SDS-PAGE and visualized by a silver staining kit (Bio-Rad) according to the manufacturer's protocol.

**Results**

***ADP-bound Sensor I mutant loses its ability to resist digest by trypsin***

ATP hydrolysis by human GST-hCdc6 WT induces a conformational change as assayed by partial tryptic digest (Herbig et al. 1999). GST-hCdc6 WT bound to ATP, ATPγS, or UTP was completely digested by trypsin, while ADP inhibited trypsin digestion (Figure 33a). Herbig et al. (1999) report that the Walker A mutant of hCdc6 was unable to be stabilized by nucleotide binding (Figure 33b). The Walker B mutant, however, was stabilized against trypsin action by binding to ATP, ATPγS, and ADP (Herbig et al. 1999) (Figure 33c), indicating that the mutation locked hCdc6 in a conformation

resembling post ATP hydrolysis of the wild type protein. ADP release might be necessary for hCdc6 return to the original conformation.

The Sensor I motif allows proteins to distinguish ATP from ADP binding (Liu et al. 2000). Since ATP hydrolysis is required for each MCM loading event by hCdc6 (Frolova et al. 2002), we can speculate that upon hydrolysis, hCdc6 undergoes a conformational change, mediated by the Sensor I motif that signals completion of its action and possibly unmasks the nuclear export signal. To evaluate the ability of the Sensor I mutant to undergo a conformational change, we employed a partial tryptic digest assay (Herbig et al. 1999).

Substituting negatively charged amino acids for serine or threonine residues that undergo phosphorylation in vivo can mimic the phosphorylated form of the protein. Pseudo-phosphorylated human GST-hCdc6 mutant proteins were assayed for their ability to undergo an ADP-induced conformational change. Recombinant proteins with primary phosphorylation sites at positions S54, S74, and S106 (3XD) mutated to aspartic acid as well as mutant proteins with all putative phosphorylation sites (Figure 32) replaced with aspartic acid do not lose their ability to undergo conformational change upon ADP binding in this assay. These mutants do not exhibit trypsin digest protection when bound to ATP, ATP$\gamma$S, or UTP. As expected, Sensor I mutants were unable to exhibit protection from trypsin action when incubated with ADP (Figure 34).

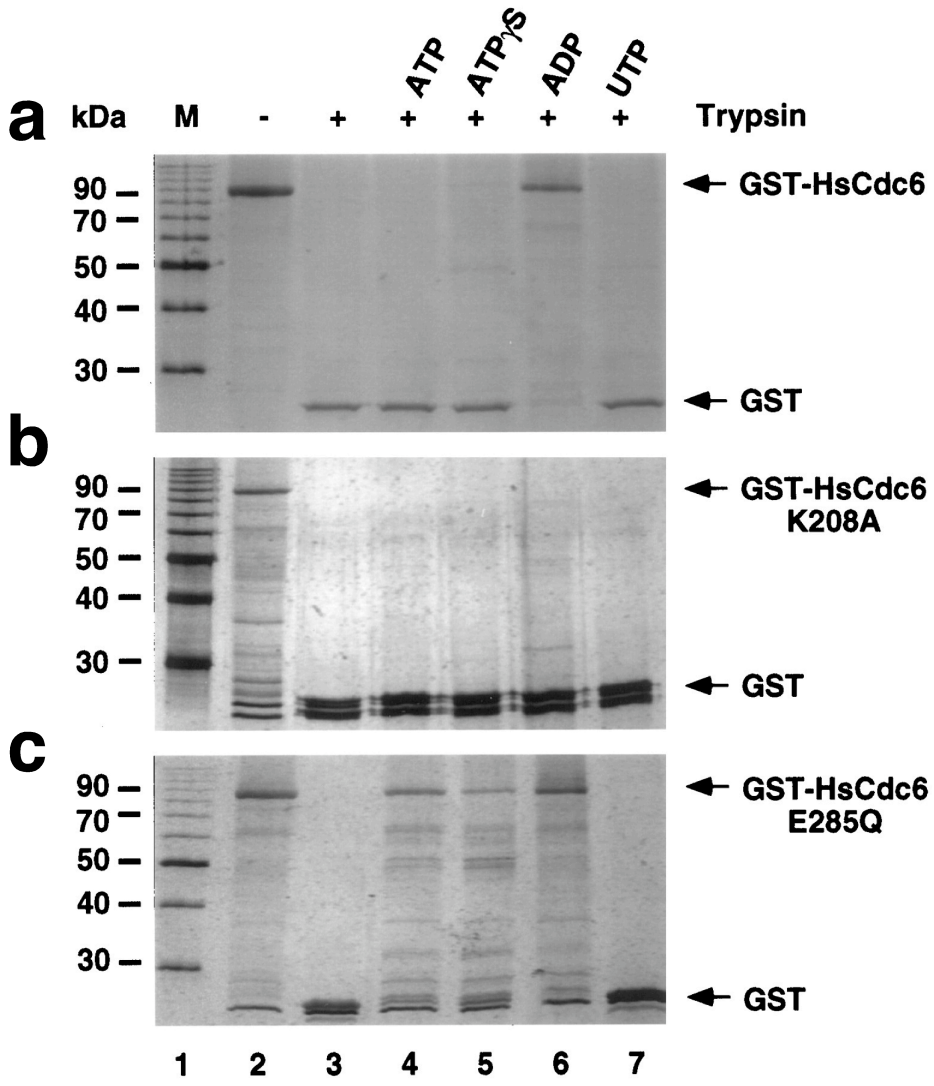


**Figure 33. Partial tryptic digest analysis of wild type and mutants of GST-hCdc6.** (**a**) Partial tryptic digest of human GST-hCdc6 wild type and the Walker motif mutants. Wild type hCdc6 is stabilized against digest by trypsin when incubated with ADP, but not ATP, ATPγS, or UTP. (**b**) The Walker A (K208A) mutant fails to exhibit such protection by ADP binding, while (**c**) the Walker B (E285Q) mutant of human GST-Cdc6 is protected by binding of either ATP, ATPγS, or ADP, but not UTP. Figure adapted from Herbig *et al.*, 1999.

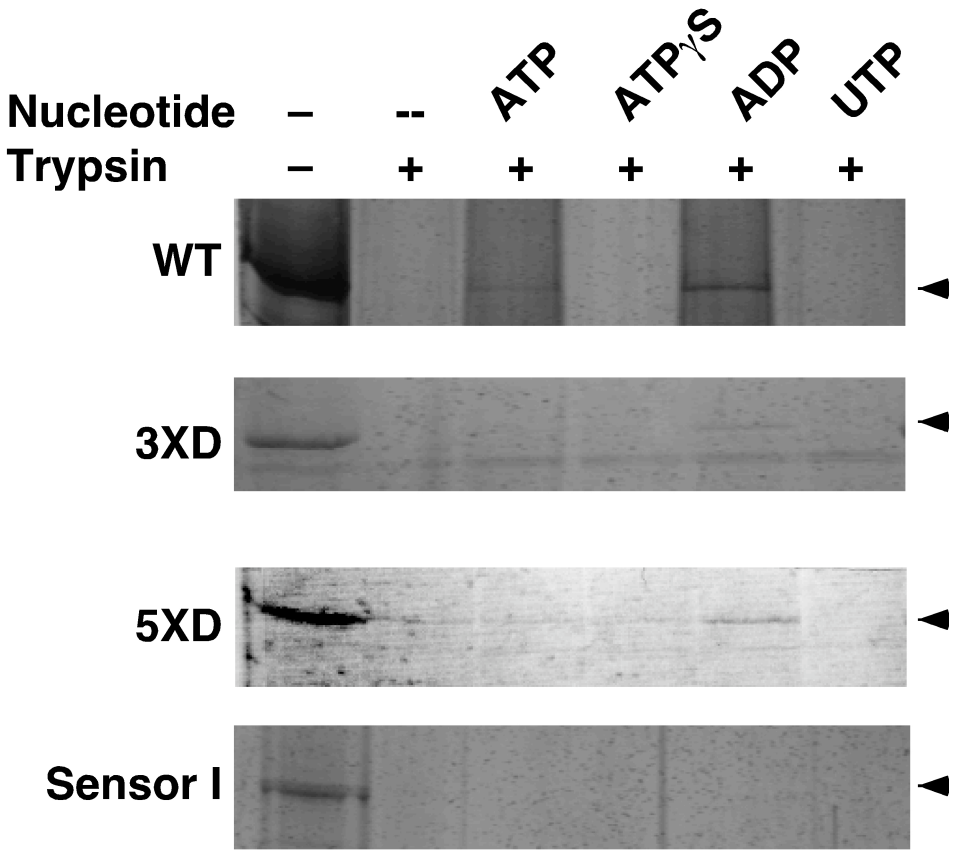


**Figure 34. A partial tryptic digest of wild type, pseudophosphorylated, and Sensor I GST-hCdc6.** Wild type and pseudophosphorylated mutants of GST-hCdc6 are stabilized against tryptic digest by incubation with ADP, but not ATP, ATPγS, or UTP. Sensor I motif mutant protein, however, is unable to be stabilized by any of the nucleotides present in the reaction mixture.

***HCdc6 is gradually exported from the nucleus in the S phase of cell cycle***

A current model (Figure 31a) of initiation of DNA replication predicts that hCdc6 export occurs *en masse* immediately after the MCM loading, but before unwinding, priming, or DNA synthesis. Previous studies reported that hCdc6 localized to the nucleus in human cells synchronized with aphidicolin (Saha et al. 1998), which blocks DNA synthesis by inhibiting the replicative polymerase, but not the primase activity of polymerase-α-primase. Human cells that were blocked with thymidine or hydroxyurea exhibited cytoplasmic localization of the hCdc6 protein. Replication forks are quickly stalled by the absence of deoxyribonucleotides. Described methods describe only a static picture of hCdc6 localization at a specific time point of the cell cycle, leaving open the question of kinetics of hCdc6 nuclear export. To address the question of whether hCdc6 is gradually released during S phase or exits *en mass* as cells transition from G1 to S, we decided to monitor the sub-cellular localization of hCdc6 in HeLa cells as they enter the cell cycle after a mitotic block. To assay the kinetics of sub-cellular location of human Cdc6, we created an N-terminal GFP fusion of the protein and observed its location as cells progressed through S phase of the cell cycle.

In order to validate the GFP-tagged human Cdc6 as a system to assay its sub-cellular location we tested its location under conditions used previously with untagged Cdc6. Earlier studies revealed that wt Cdc6 localizes to the nucleus in G1 and to the cytoplasm in S phase of the cell cycle. In addition, Cdc6 was found in the nucleus of cells blocked with aphidicolin, but the protein was cytoplasmic in cells blocked with hydroxyurea or thymidine (Saha et al. 1998; Jiang et al. 1999; Petersen et al. 1999). Electroporation of asynchronous U2OS cells and injection of exponentially growing

HeLa cells were used to show that GFP-hCdc6 WT did display cellular localization pattern similar to endogenous protein (results not shown). In addition, GFP-hCdc6 localized in cells blocked with aphidicolin and thymidine to the same cellular compartment as reported previously (results not shown). Thus, we concluded that microinjection of GFP-tagged Cdc6 is a valid system to monitor its sub-cellular localization.

BrdU incorporation establishes the time of S phase beginning in HeLa cells as they are released from a nocodazole block. Nocodazole is a microtubule inhibitor and synchronizes cells in G2/M. Previous studies by Herbig et al. (1999) reported that HeLa cells released from a nocodazole block, began S phase at 10 hr post-release as assayed by flow cytometry (Figure 35a). In agreement with these results, HeLa cells began BrdU incorporation after 10 hr following release from a nocodazole block (Figure 35b). Since BrdU incorporation was monitored during 2 hr pulses, the percentage of cells positive for BrdU reflects cells actively replicating at a given time point.

Kinetic analysis of hCdc6 export from the nucleus was conducted by injecting plasmid DNA encoding GFP-hCdc6 into HeLa cells synchronized with nocodazole at 5 to 8 hr after release from nocodazole block. After injection, BrdU was added to the medium. GFP fusion protein was detected in the nucleus by fluorescent microscopy analysis at 2 hr after injection (data not shown). At 10 hrs after nocodazole release, the number of cells with hCdc6 in the cytoplasm gradually increased, while the population of cells with hCdc6 found in the nucleus was reduced (Figure 36b).

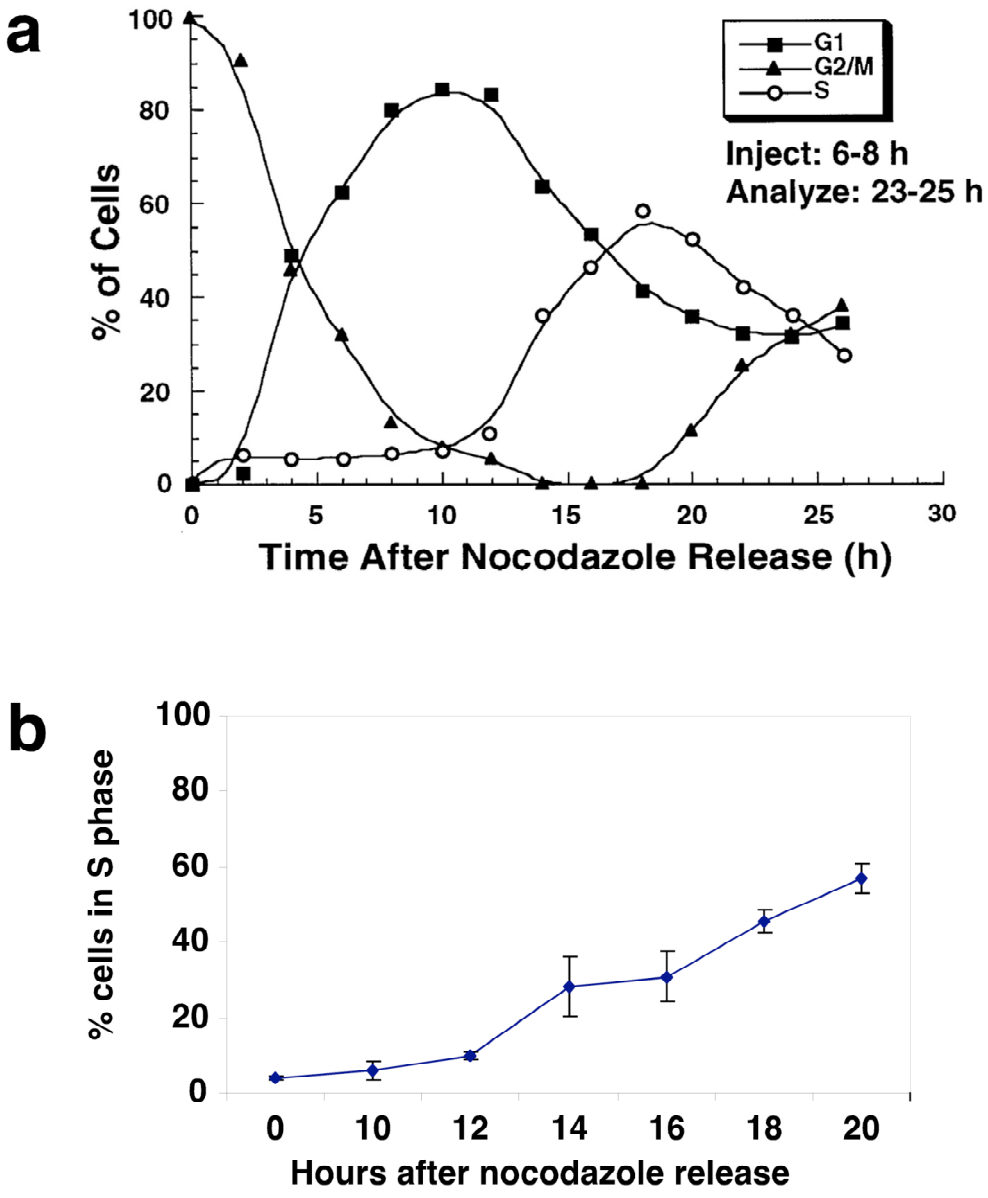


**Figure 35. Kinetics of GFP-hCdc6 nuclear export in synchronized HeLa cells.** (**a**) Cell cycle analysis of DNA content of HeLa-S3 cells blocked by nocodazole and released into drug free media as assayed by flow cytometry (Adapted from Herbig *et al.*, 1999). (**b**) Kinetic analysis of non-injected HeLa cells synchronized at G2/M with nocodazole and released into G1. DNA replication was assessed by BrdU incorporation in 2 hr pulses.

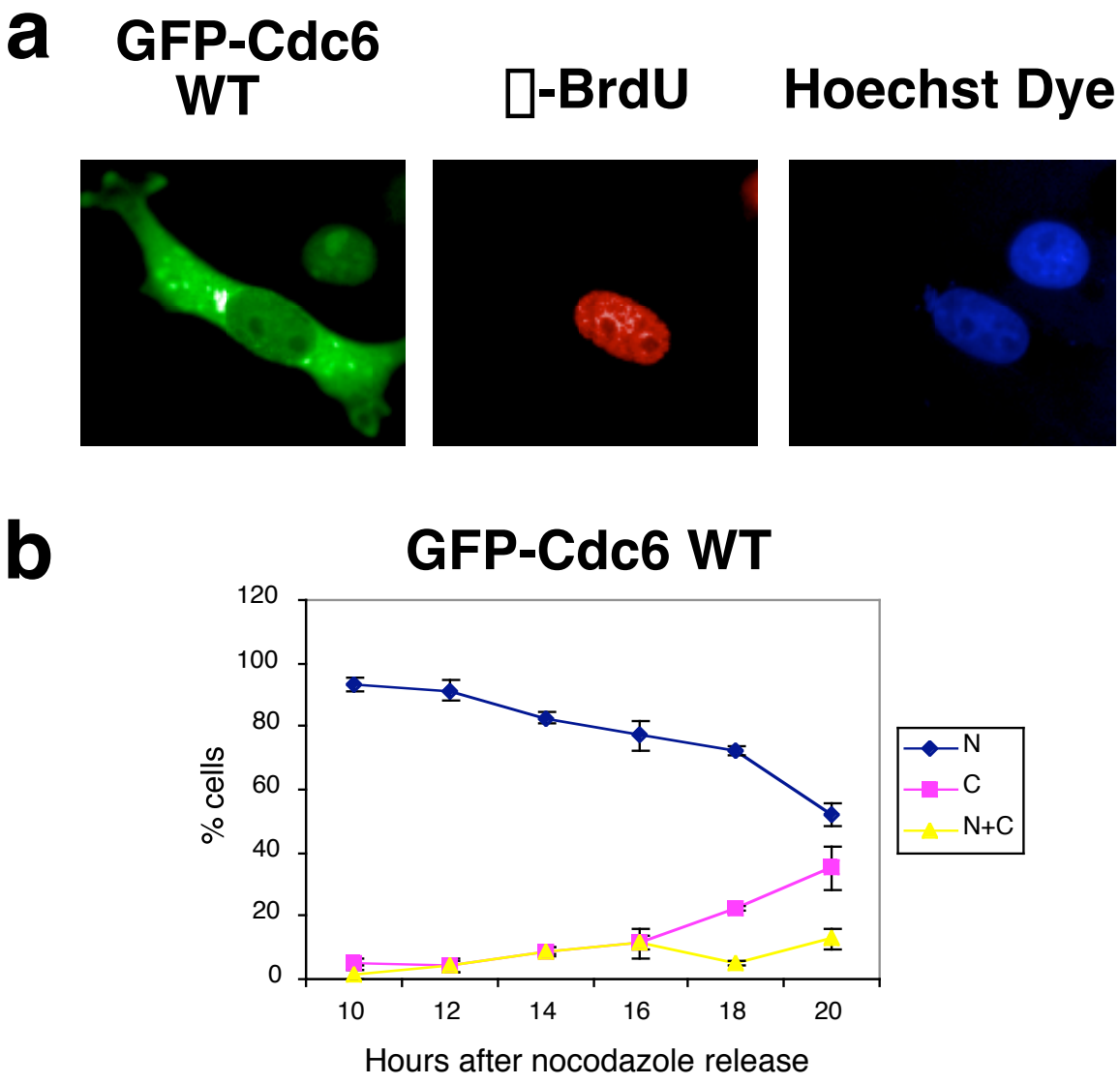


**Figure 36. Kinetic analysis of GFP-hCdc6 WT sub-cellular localization and DNA replication in synchronized HeLa cells.** (**a**) GFP-hCdc6 WT protein does not inhibit DNA replication when localized to the cytoplasm. BrdU incorporation was conducted in 2 hr pulses. GFP-fused human Cdc6 protein was injected in early G1 into synchronized HeLa cells. (**b**) GFP-hCdc6 WT plasmid DNA was injected into HeLa cells in early G1. GFP positive cells were assayed for sub-cellular localization of protein at indicated time points (N-nuclear, C-cytoplasmic, N+C-protein is present in both nucleus and cytoplasm). The results represent four independent experiments with an average number of cells at each time point ranging from 49 to 90.

Concomitant staining with BrdU indicates that cells containing cytoplasmic GFP-hCdc6 were able to replicate DNA, but a small population of GFP-hCdc6 that localized to the nucleus in S phase constitutively inhibited DNA replication (Figure 36a).

***GFP-hCdc6 Walker B mutant delays S phase and blocks DNA replication***

Herbig et al. (1999) reported that the Walker B mutant of hCdc6 impaired or inhibited DNA replication when GST-hCdc6 Walker B recombinant protein was microinjected into synchronized HeLa cells in early G1. We tested the GFP-hCdc6 Walker B mutant to verify that it retains the dominant-negative phenotype of the GST-hCdc6 Walker B mutant in DNA replication from 10 to 20 hrs after release from nocodazole block, consistent with the failure of the cell to undergo DNA replication. The protein was found constitutively in the nucleus even at 20 hr after release from nocodazole (Figure 37b). To test the ability of GFP-hCdc6 Walker B mutant protein to inhibit DNA synthesis in HeLa cells, as shown before for the GST fused protein, DNA replication was assessed by BrdU incorporation assay. Fluorescence microscopy revealed strong inhibition of DNA replication by GFP-hCdc6 Walker B mutant protein in microinjected cells (Figure 37a). These results argue that the GFP mutant protein, like the GST mutant protein, had a dominant-negative phenotype in human cells.

***Human GFP-Cdc6 Sensor I mutant behaves like Walker B mutant***

Schepers et al. (2001) in their analysis of the Sensor domain mutants of Cdc6 reported that the phenotypes of Walker B and Sensor I mutants in yeast Cdc6 were

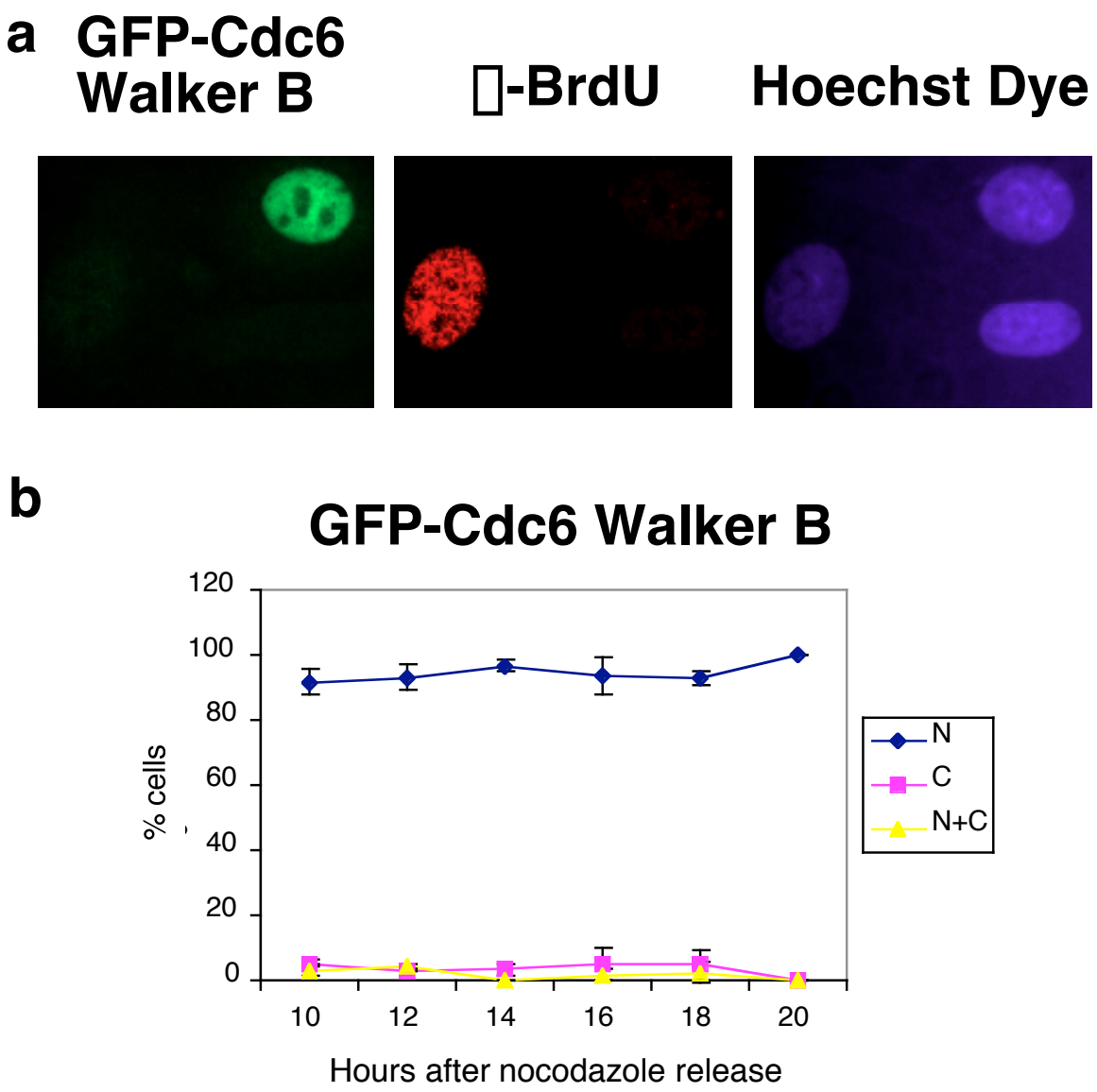


**Figure 37. GFP-hCdc6 Walker B is constitutively nuclear and unable to function in DNA replication.** (**a**) The GFP-hCdc6 Walker B mutant was assessed for its ability to participate in DNA replication. Analysis was conducted under the same conditions as the kinetic quantification experiment except that BrdU was added for 2 hrs at 18 and 20 hr time points. Non-injected cells undergoing replication at these time points averaged 70%. (**b**) Kinetic analysis of GFP-hCdc6 Walker B localization in synchronized HeLa cells. GFP positive cells were assayed for sub-cellular localization (N-nuclear, C-cytoplasmic, N+C-protein is present in both nucleus and cytoplasm). The results represent three independent experiments with the number of cells analyzed at each time point ranging from 24 to 70.

indistinguishable. Both mutants exhibited a dominant negative phenotype, delayed S phase progression, and were unable to efficiently load MCM or initiate DNA replication. Importantly, Sensor I mutants were shown to be more stable than the wild type protein.

Having verified that the GFP-fused Walker B mutant of human Cdc6 behaves similarly in its ability to delay S phase progression and inhibit DNA replication, we analyzed the GFP-hCdc6 Sensor I mutant protein for the same properties. As expected, the GFP-fused Sensor I mutant of human Cdc6 was exclusively nuclear in S phase of the cell cycle (Figure 38b). In addition, the GFP-hCdc6 mutant efficiently inhibited replication when over-expressed in human cells (Figure 38a).

***Phosphorylation alone is not sufficient for hCdc6 nuclear export***

It has been reported that phosphorylation of Cdc6 is required for its export from Xenopus nuclei replicating in egg extracts (Pelizon et al. 2000) and from human nuclei (Jiang et al. 1999; Petersen et al. 1999). However, it has not been conclusively established that phosphorylation alone is sufficient for Cdc6 export in either system. Several groups reported that pseudo-phosphorylated Cdc6, in which three or four serine phosphorylation sites were replaced with aspartic acid, was found predominantly in the cytoplasm of exponentially growing cells (Jiang et al. 1999; Petersen et al. 1999; Delmolino et al. 2001). The possibility that pseudo-phosphorylated mutants cannot be imported into the nucleus was ruled out. None of the groups analyzed the mutants with all phosphorylation sites mutated to aspartic acid.

We analyzed sub-cellular localization of the 5XD (S45D, S54D, T67D, S74D,

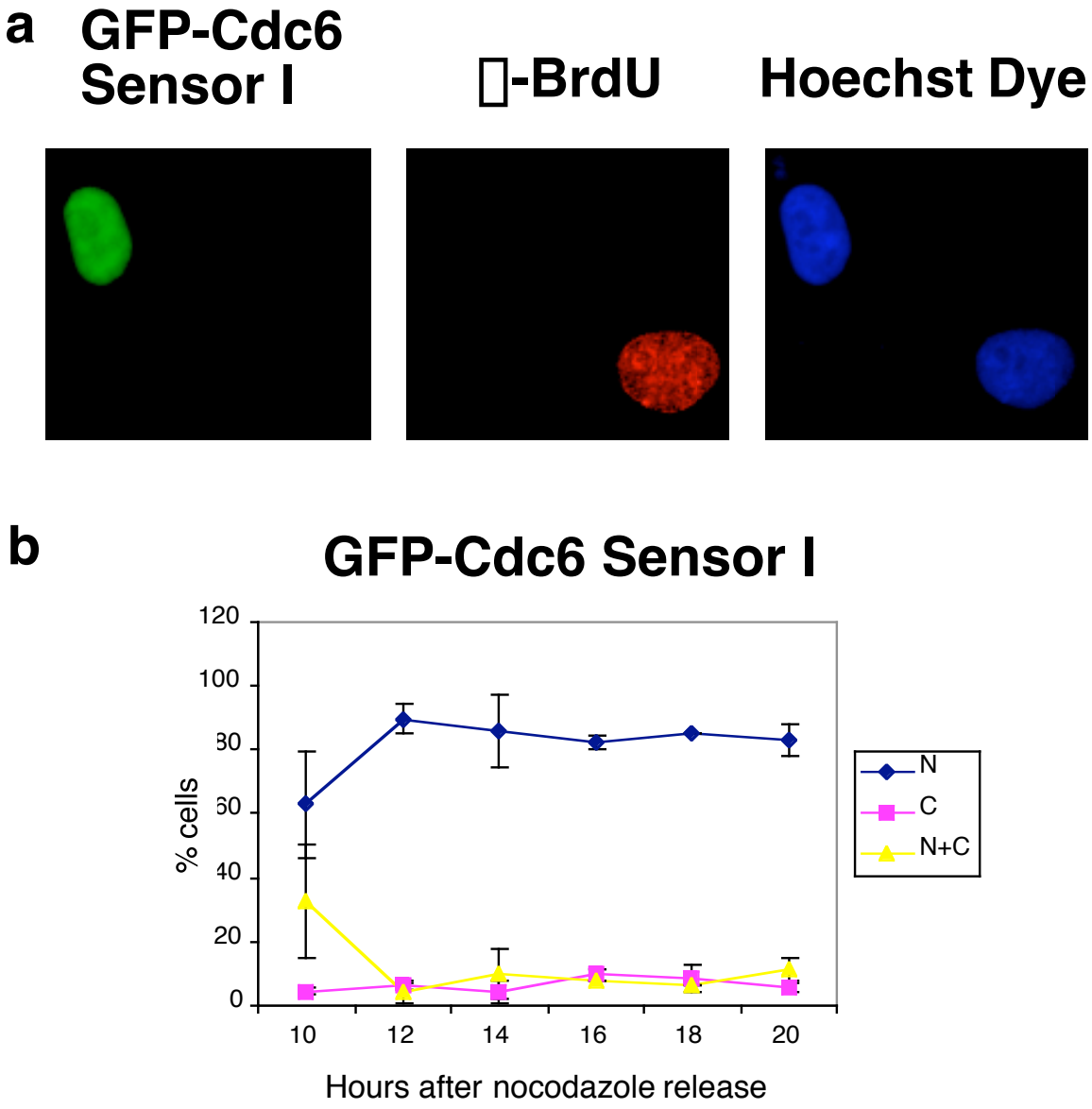


**Figure 38. The Sensor I mutant of hCdc6 is found constitutively in the nucleus and inhibits cellular DNA replication.** (**a**) The GFP-hCdc6 Sensor I mutant inhibits cellular DNA replication. Replicating cells were determined by incubating HeLa cells for 2 hr in BrdU containing medium. (**b**) Kinetic analysis of the GFP-hCdc6 Sensor I mutant. GFP positive cells were assessed for sub-cellular localization of the protein (N-nuclear, C-cytoplasmic, N+C-protein is present in both nucleus and cytoplasm). The results represent two independent experiments with an average number of cells analyzed at each time point ranging from 27 to 109.

S106D) mutant, expressed in G1 as a GFP fusion protein as cells progress from G1 to S. Under these conditions, 5XD mutant protein remained nuclear even at 20 hrs post-nocodazole release, indicating an inability of the protein to exit the nucleus in S phase (Figure 39a). Cells that expressed the 5XD mutant protein in the nucleus were BrdU negative, indicating an inability to replicate DNA. These data allow us to conclude that cells expressing wt or mutant GFP-Cdc6 in the nucleus are unable to support replication, therefore Cdc6 export from the nucleus is required for the initiation of chromosomal replication. Results of this study as well as previous studies suggest that nuclear export depends on phosphorylation of Cdc6. Since ATP hydrolysis has been shown to be required for MCM loading by Cdc6 and the Sensor I motif might mediate a conformational change resulting from this catalytic activity, we speculated that a conformational change together with the phosphorylation of Cdc6 are required for the protein's export from the nucleus. However, when the Sensor I mutation was combined with the pseudophosphorylated 5XD or 3XD mutations, the GFP fusion proteins were found constitutively in the cytoplasm (Figure 39b,c)

***Sensor I/Pseudophosphorylated mutants of HCdc6 are unable to enter the nucleus***

Since the pseudophosphorylated/Sensor I double mutant proteins were constitutively found in the cytoplasm, both in G1 and S, it is possible that they enter the nucleus and shuttle quickly to the cytoplasm. Alternatively, the double mutant proteins may not be imported into the nucleus because their nuclear localization signal is masked or

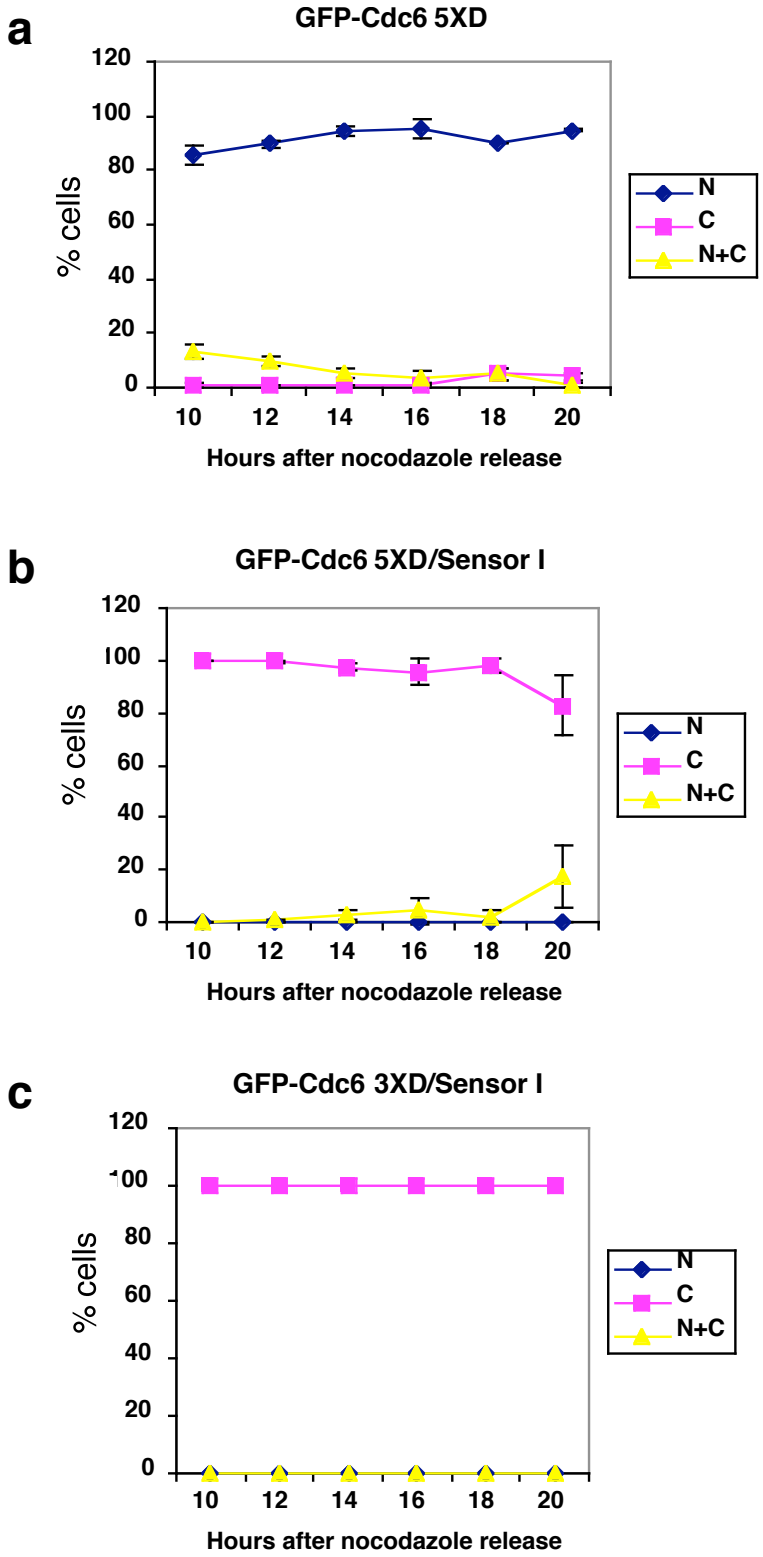


**Figure 39. Mutants of GFP-hCdc6 exhibit defects in sub-cellular localization.** (**a** ) Kinetic analysis of GFP-hCdc6 5XD mutant. The mutant protein is consitutively found in the nucleus when expressed in human cells in S phase. The results represent two independent experiments with a number of cells analyzed at each time point ranging from 19 to 130. (**b**) Kinetic analysis of GFP-hCdc6 5XD/Sensor I double mutant. GFP positive cells were assessed for sub-cellular localization of the protein (N-nuclear, C-cytoplasmic, N+C-protein is present in both nucleus and cytoplasm). The results represent two independent experiments with a number of cells analyzed at each time point ranging from 27 to 106. (**c**) Kinetic analysis of GFP-hCdc6 3XD/Sensor I double mutant. GFP positive cells were assessed for sub-cellular localization of the protein (N-nuclear, C-cytoplasmic, N+C-protein is present in both nucleus and cytoplasm). The results represent one experiment with a number of cells analyzed at each time point ranging from 50 to 117.

inactivated by the mutations. To determine whether the proteins are able to enter the nucleus, we have employed a specific Crm1-inhibiting agent Leptomycin B (LMB). This cytotoxin disrupts the formation of the Ran (GTP)-Crm1 transport complex, thus blocking Crm1-mediated export from the nucleus to the cytoplasm (Nishi et al. 1994). hCdc6 export from the nucleus in human cells has been shown to be mediated through the Crm1 pathway (Jiang et al. 1999) and was blocked by LMB.

GFP-hCdc6 WT was used as a control to establish the protocol. HeLa cells were blocked with nocodazole and released into the cell cycle. At 4-5 hours after release, cells were injected. Seven hours after nocodazole release, cells were harvested to determine the localization of GFP-hCdc6 WT. Concurrently Leptomycin B was added to the other dishes, which were then incubated until 20 hrs after nocodazole release, when cells were expected to be in S phase or G2. At seven hours after release, cells were still in G1 and the majority of cells showed that GFP-hCdc6 was in the nucleus (Figure 40a). At 20 hrs post-release the majority of cells had GFP-hCdc6 in the cytoplasm (Figure 40a). However, in LMB treated cells, the overwhelming majority of cells showed GFP-hCdc6 in the nucleus at 20 hr time point, indicating that a block to Crm1-mediated nuclear export had occurred (Figure 40a). When the sub-cellular location of GFP fusion proteins containing the 3XD/Sensor I and 5XD/Sensor I double mutations was tested in the presence of LMB as described above, all of the cells contained exclusively cytoplasmic GFP fusion proteins (Figure 40b). This result indicates that these mutants were not able to enter the nucleus.

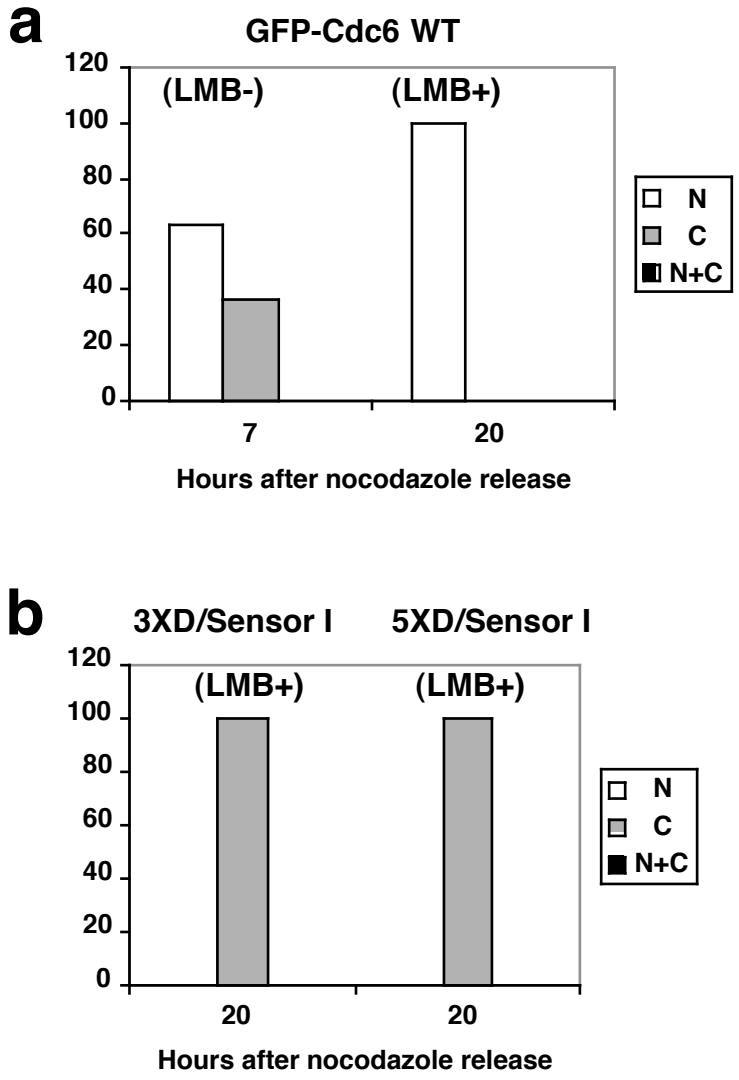


**Figure 40. The pseudo-phosphorylated Sensor I double mutants of hCdc6 are unable to enter the nucleus.** (**a**) GFP-hCdc6 WT was injected into synchronized HeLa cells in early G1. Cells were fixed and analyzed at 7 hrs post release, when cells are in G1 and at 20 hr, when cells are finishing S phase. LMB was added to the 20 hr sample at 7 hrs post nocodazole release. (**b**) Analysis of 3XD/Sensor I and 5XD/Sensor I mutants of GFP-hCdc6 under the same protocol as in panel a.

***T67-P68 mutation causes a defect in hCdc6 sub-cellular localization***

Previous analysis of 3X ((Saha et al. 1998) and 4X (Delmolino et al. 2001) phosphorylation mutants of hCdc6 indicated no functional difference between these mutants in sub-cellular localization. Non-phosphorylatable alanine mutants were consistently found in the nucleus of transfected cells, while pseudo-phoshorylated aspartic acid mutants localized predominantly to the cytoplasm. Putative posphorylation site T67 was omitted from analysis in both 3X and 4X mutants in those studies. However, comparing the localization pattern of 3XD/4XD and 5XD mutants of hCdc6 reveals a significant difference elicited by a mutation at threonine in the position 67 (compare the data in Delmolino *et al*. to Figure 39). A number of studies have suggested that certain prolylic bonds might undergo isomerization, which plays a role in regulation of protein function (Schutkowski et al. 1998; Fischer 2000). These studies also indicate that isomerization is dependent upon phosphorylation of the threonine preceding proline in the primary structure. Chemical properties of serine are distinct from those of threonine, making the latter a better substrate for isomerase action (Ranganathan et al. 1997; Fischer 2000; Wintjens et al. 2001). We speculated that mutating T67 to alanine might have affected the integrity of the prolylic bond necessary for isomerization reaction. Thus, we decided to investigate a possibility that the T67-P68 peptide bond might play a role in the regulation of hCdc6 function and/or localization by mutating proline P68.

A mutant of hCdc6 was created in which proline in position 68 was replaced with alanine, while T67 was retained. The mutant was analyzed as a GFP fusion protein for its

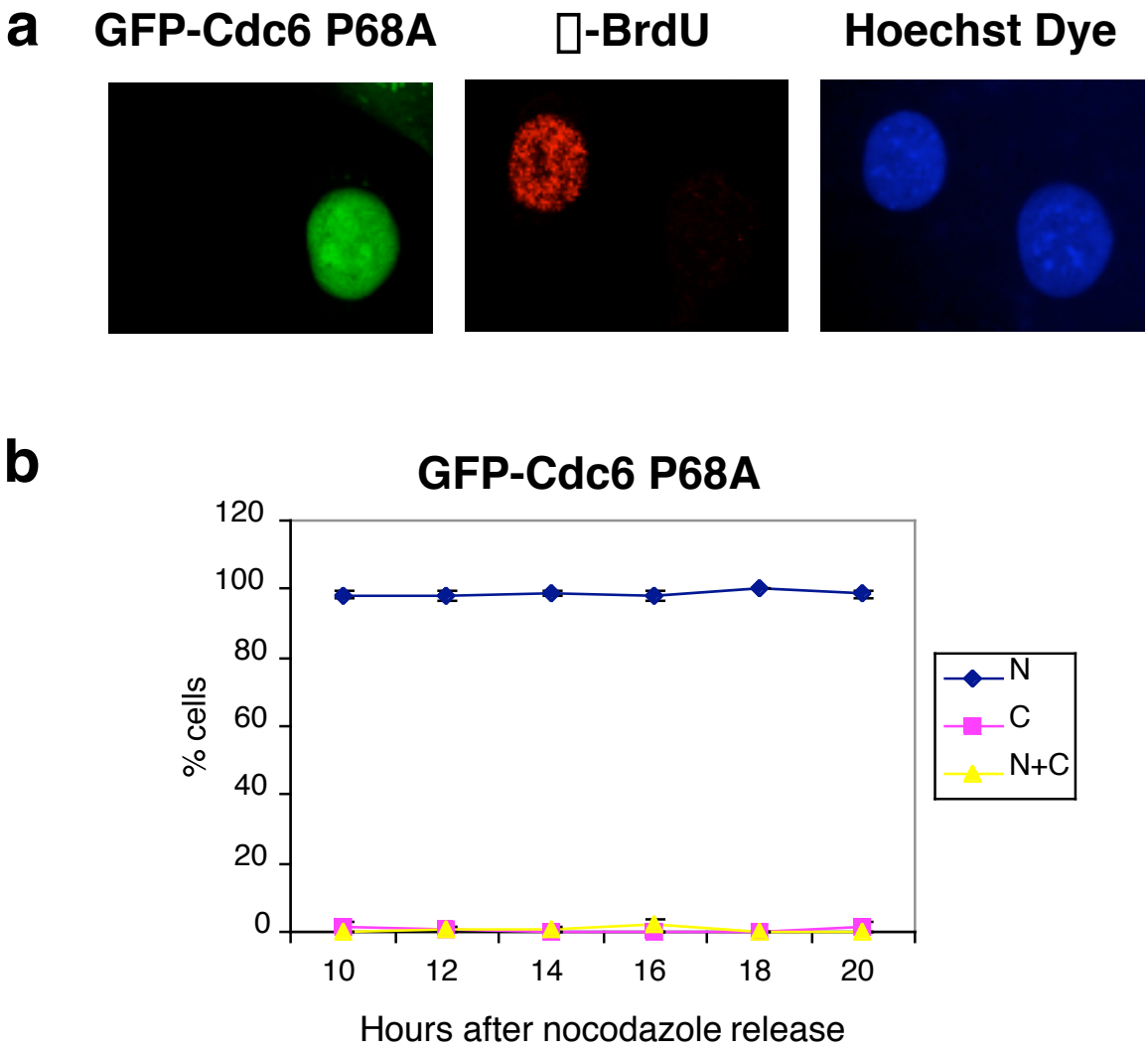


**Figure 41. The GFP-hCdc6 P68A mutant is located predominantly in the nucleus and fails to replicate DNA.** (**a**) The GFP-hCdc6 P68A mutant inhibits cellular DNA replication in synchronized HeLa cells at time points corresponding to late S phase. (**b**) The GFP-hCdc6 P68A mutant is nuclear even at 20 hr after HeLa cells were released after nocodazole block. The results represent two independent experiments with 42 to 116 cells analyzed at each time point.

sub-cellular localization during G1 and S phase as described above. Results indicate that GFP-hCdc6 P68A mutant was found constitutively nuclear in this assay (Figure 41b). In addition, this mutant blocked cellular DNA replication when expressed in HeLa cells in G1 (Figure 41a).

**Discussion**

A number of studies have conclusively shown that Cdc6 is exported to the cytoplasm apparently after its function is completed (Saha et al. 1998; Jiang et al. 1999; Petersen et al. 1999). These studies were conducted by blocking cells at different points in the cell cycle using pharmaceutical agents. However, this approach did not shed light on the kinetics of this export.

Results of this study suggest that hCdc6 is being gradually exported from the nucleus (Figure 35b). These data might suggest that hCdc6 export depends on firing of a particular origin. As origins fire during S phase, Cdc6 is released starting from the early origins to late ones, which would explain gradual release of hCdc6 from the nucleus. One of the predictions of this hypothesis would be an observation that hCdc6 should be present on chromatin at a late origin while absent at an early origin after an early origin fires. This hypothesis still needs to be addressed in future studies, possibly by conducting chromatin immunoprecipitation assays, comparing hCdc6 presence at an early and a late origin as a cell progresses through S phase.

Phosphorylation is required for hCdc6 export. In this study, however, we established that the pseudo-phosphorylated mutant of hCdc6 with aspartate replacing all five putative phosphorylation sites fails to be exported from the nucleus, while the 4XD

mutant, in which T67 residue is unaffected by a mutation, can be exported from the nucleus. The present study, therefore, suggests that in order to be exported, hCdc6 must not only be phosphorylated, but should also receive a secondary signal indicating that its function is complete. Several explanations could be offered concerning the nature of such a signal. A number of studies including this one indicated that the Sensor I motif is required for the proper functioning of hCdc6 (Schepers and Diffley 2001; Takahashi et al. 2002). Since Sensor I allows hCdc6 to distinguish ADP from ATP bound forms of nucleotide, we hypothesize that upon MCM loading, hCdc6 hydrolyzes ATP and consequently changes its conformation. Such a conformational change might lead to the exposure of the nuclear export signal. This conformational change is evident from analysis of partial tryptic digests of the wild type protein bound to ADP ((Herbig et al. 1999). Conversely, the Sensor I mutant is unable to undergo this conformational change and provide the secondary signal for hCdc6 export. Inability to change conformation would explain the failure of the Sensor I mutant protein to exit the nucleus in S phase.

An additional explanation for hCdc6 translocation control can be based on possible prolylic bond isomerization as suggested by the analysis of the P68A mutant. We propose that upon completion of its primary function, hCdc6 is phosphorylated on threonine 67 and consequently acted upon by a threonine-proline isomerase, for example human Pin1 (Lu 2004; Lim and Ping Lu 2005). Conformational changes elicited by the action of this hypothetical isomerase would unmask the nuclear export signal, possibly near the Sensor I motif, which contains a putative nuclear export signal (Figure 32). Phosphopeptide mapping indicated that only S54, S74, and S106 are stably phosphorylated *in vivo* (Jiang et al. 1999; Petersen et al. 1999). However, the analyses in

those experiments were done on asynchronous cell populations. Our proposed mechanism would suggest a very precise timing of phosphorylation and its possibly transient nature. Therefore, such phosphorylation might not be detected in an asynchronous pool of cells. In order to investigate our hypothesis further, more direct involvement of an isomerase needs to be established. This hypothesis would explain the failure of the 5XD mutant to exit the nucleus. Analysis of Pin1 interaction with phosphorylated and pseudo-phosphorylated substrates indicated that aspartic acid substitution to mimic the phosphorylated state of a protein provides a poor substrate to the isomerase, since it provides only a single anionic charge. True phosphorylation provides di-anionic charge, which may be required for the proper interaction with the Pin1 isomerase (Fischer 2000).

Cyclin dependent kinases (CDKs) were implicated in the phosphorylation of hCdc6 (Petersen et al. 1999; Herbig et al. 2000; Coverley et al. 2002). Cyclin A/CDK2 and Cyclin E/Cdk2 complexes were able to phosphorylate hCdc6 *in vitro* (Herbig et al. 2000). We speculate that another kinase, acting later in the cell cycle than CDKs, could transiently phosphorylate hCdc6. One of the candidates to consider can be the Cdc7/Dbf4 complex that acts after CDK action but before Cdc45 loading on chromatin. There is, however, a discrepancy in the sequence of CDK and Cdc7/Dbf4 action, which needs to be resolved (Bell and Dutta 2002). Alternatively, another yet not identified kinase might perform the phosphorylation.

We, therefore, propose a model (Figure 42) in which mammalian Cdc6 is recruited to chromatin by ORC subunits and together with Cdt1 load the MCM 2-7 complex onto chromatin. In order to complete its function of loading the MCM 2-7

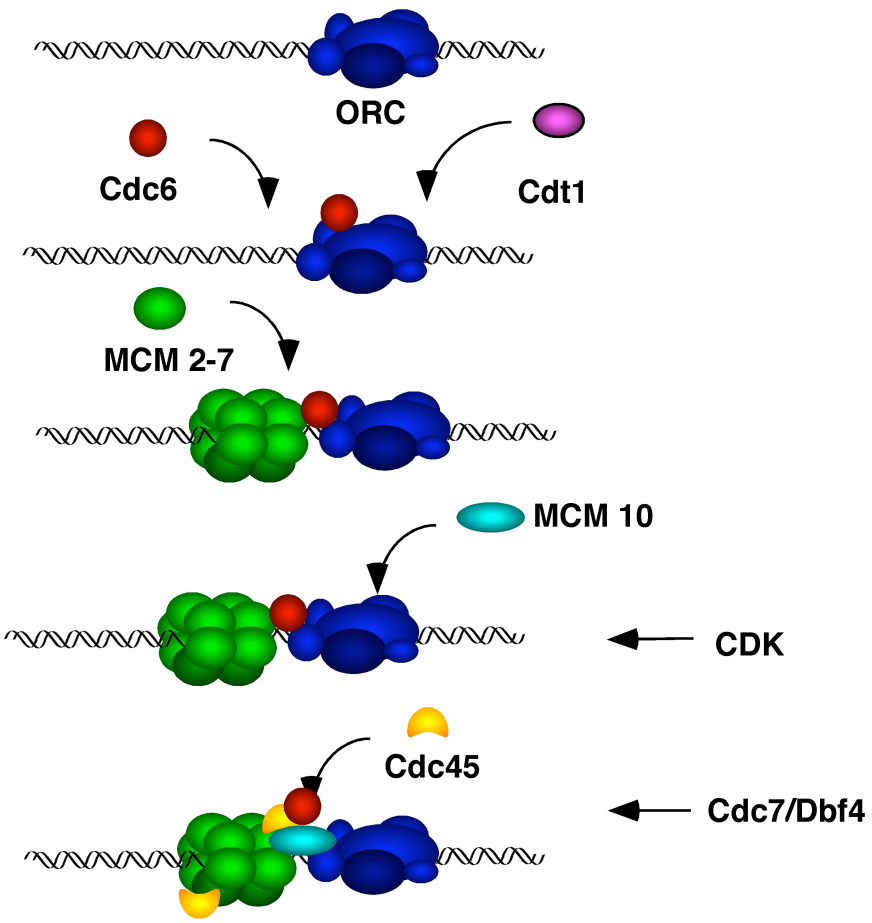


**Figure 42. A suggested model for the timing and role of phosphorylation and ATP hydrolysis of human Cdc6 protein in initiation of DNA replication.**

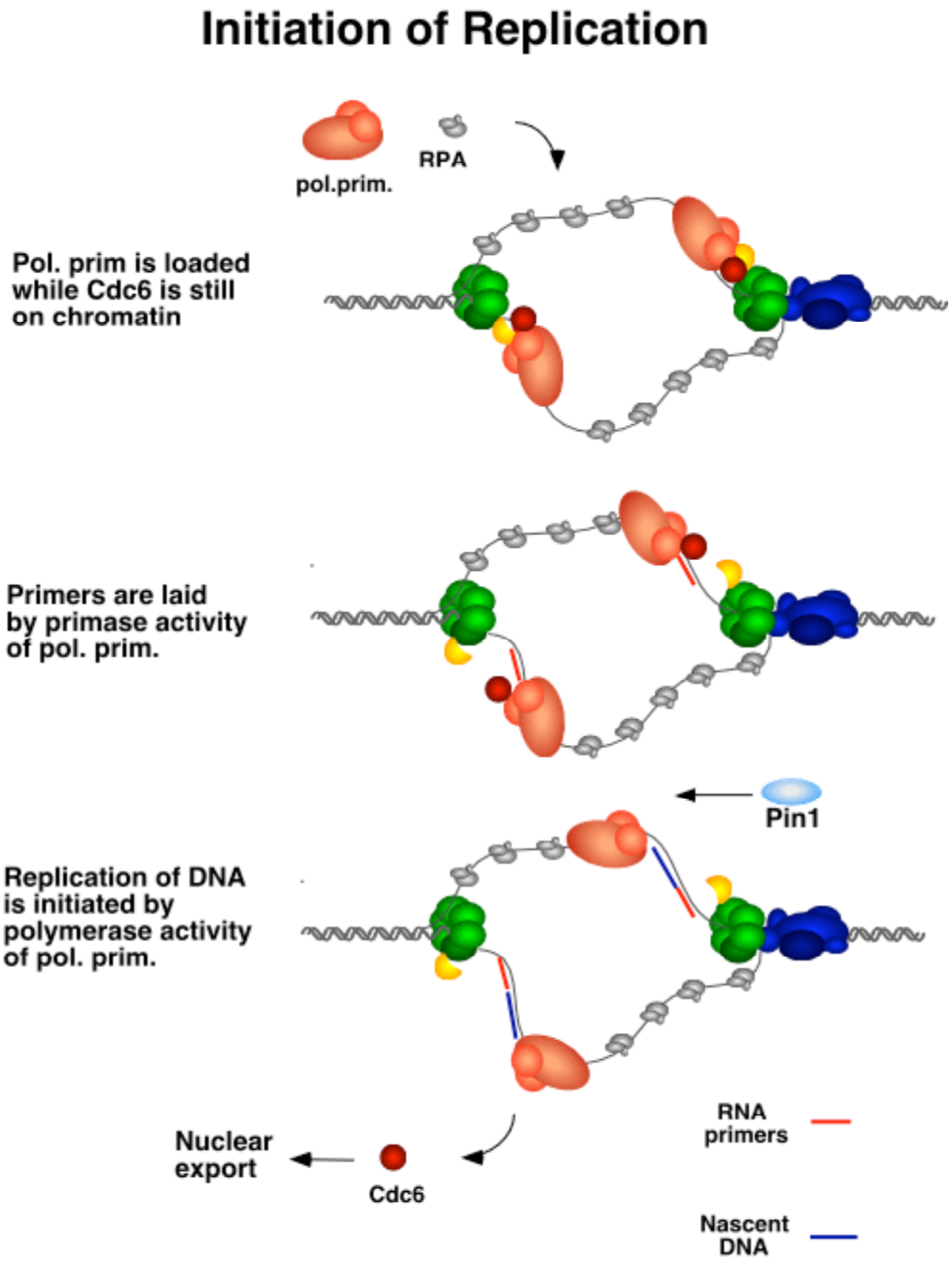


**Figure 43. A putative model suggesting possible mechanism of human Cdc6 export from the nucleus and the role of human Cdc6 in initiation of DNA replication.** Not all the components of cellular machinery are presented in the model, but only those relevant for the proposed mechanism of action. The sequence of phosphorylation of human Cdc6 and ATP hydrolysis has not been conclusively established. Thus, the sequence of these events is tentative.

complex, Cdc6 hydrolyzes ATP to ADP which remains tightly bound (Liu et al. 2000). The ADP-bound Cdc6 undergoes a conformational change, mediated by the Sensor domains. However, mammalian Cdc6 stays in the nucleus and is phosphorylated by CDK. Phosphorylation alone is not sufficient for the export from the nucleus. Mammalian Cdc6 might later be transiently phosphorylated at T67 and undergo an additional conformational change around the P68 prolylic bond due to the action of an isomerase, which provides the release signal for Cdc6 export from the nucleus (Figure 43). Due to the lack of data, the sequence of events is tentative and could be modified as more information becomes available.

# CHAPTER IV

## SUMMARY AND CONCLUSION

The field of DNA replication has experienced a renaissance in the recent years. Since the structure of DNA molecule was deciphered, some of the greatest minds of the scientific world have tried to unravel the molecular mechanism of DNA replication. Different approaches and study systems were employed through the years to identify replication factors and their function in chromosome doubling. Much progress was made through utilization of cell-free systems from *Xenopus*, genetic manipulation in yeast, and biochemical dissection of *in vitro* replication assays. However, renewed interest was prompted by discovery of apparent links among DNA replication, recombination, and DNA repair. The focus of the research has also shifted from identifying and characterizing the components of the replisome to determining the network of interactions among the replication factors and visualizing the workings of replication engines on the atomic level through structure determination (O'Donnell et al. 2001; Fletcher et al. 2003; Li et al. 2003; Bowman et al. 2004; Gai et al. 2004; Sclafani et al. 2004; Stauffer and Chazin 2004; Bowman et al. 2005).

Taking into consideration the emerging complexity of the initiation of DNA replication, a great emphasis has been placed on using in vitro reconstituted reactions. Though very useful, the limitation of these systems lies in the cluttering due to the number of purified recombinant proteins involved. Investigation of interactions in the

primosome, thus, should be limited to as few factors as possible. Our study utilizes such a simplified model using either dsDNA plasmid containing SV40 origin or ssDNA molecule derived from the M13 virus. This approach requires only one viral protein (Tag), three cellular initiators pol-prim, RPA, and Topo I and allows an in depth view into the mechanism of viral replication and functional interactions among these proteins.

**RPA in SV40 replication**

Initiation of replication in SV40 monopolymerase system begins when Tag recognizes the four pentanucleotide repeats of the SV40 origin and assembles into a double hexamer on the dsDNA (Herendeen and Kelly 1996; Bullock 1997). The DNA is melted at the region proximal to the origin and helicase activity of Tag generates single-stranded DNA fragments that become immediately coated with RPA to prevent strand re-annealing. The mechanism of RPA binding to ssDNA is unclear, but initial data from the Fanning group and EM studies suggest that RPA is actively loaded by Tag (Hysinger, et al., in preparation; J.M. Carazo, Madrid, personal communication). The loading of RPA is apparently mediated by Tag interaction with a linker region of RPA70 that connects domains A and B. RPA blocks replication protein access to ssDNA and thus must be removed before pol-prim can start priming. We propose that this action is accomplished by Tag through its interaction with the C-terminus of RPA32 (Figure 30).

**Replication juggler**

Mounting evidence suggests that the cellular machinery does not exist as a set of pre-formed holocomplexes that are employed when necessary, but rather that a dynamic "hand off" mechanism is used for cell metabolism and chromatin maintenance (Kowalczykowski 2000; Essers et al. 2002; Rademakers et al. 2003; Stauffer and Chazin 2004). The hand off mechanism assumes the existence of a regulatory protein that in turn recruits other gene products as necessary depending on the demand and task at hand, replacing them on the DNA molecule (Yuzhakov et al. 1999a). One of the prominent examples of such "trading places on DNA" is the polymerase switching in eukaryotic DNA replication (Davey and O'Donnell 2000). Pol-prim, which initiates replication, is not a processive polymerase and needs to be replaced by a more processive pol δ. A sliding clamp PCNA protein is loaded by replication factor C, a clamp loader, which allows processive DNA synthesis by pol δ. Loading of PCNA leads to pol-prim displacement on the DNA molecule. Every step of the polymerase switch is mediated by interactions with RPA (Yuzhakov et al. 1999b). In order to achieve the dynamics of proteins trading places on DNA, their interactions should be rather weak and possibly transient in nature. Our model for Tag acting on RPA in SV40 replication suggests this kind of binding. The affinity of Tag, though detectable, is weak and could be functional only in conjunction with another binding site. This hypothesis is supported by the fact that Tag binds to intact RPA in an ELISA based assay, but not to individual subunits, suggesting that interactions with any single subunit were not strong enough to be detected by the assay, but the combined effect of RPA70 and RPA32 binding sites was sufficient (Dornreiter et al. 1992). The linker region that connects OBD A and OBD B of RPA70 is

an excellent candidate for this role. Preliminary results obtained by E. Hysinger in Fanning lab indicate that RPA with a charge-reversal point mutation in the linker region is defective in SV40 DNA replication. RPA70 has shown that it interacts with Tag-OBD through the region overlapping with the linker region (Iftode et al.,1999). Unpublished results from the Chazin group corroborate this observation using NMR and indicate that RPA70/Tag interactions are weak, but stronger than RPA32C/Tag, and are electrostatic in nature.

Another mechanism that utilizes RPA as a mediator of protein complex hand off is the eukaryotic nucleotide excision repair (NER) mechanism (Riedl et al. 2003). After the DNA damage is recognized by XPC/hHR23B/Centrin 2 heterotrimer and dsDNA is unwound by XPB/XPD dimer helicase, XPA and RPA interaction leads to the recruitment of a cascade of downstream repair factors, such as ERCC1 and XPG. After excision repair is complete, RPA remains on DNA to facilitate recruitment of replicative factors RFC, PCNA, pol □, and DNA ligase, which mediate gap filling.

Recent data indicates that RPA plays an active role in the recruitment of a number of proteins to DNA to sense DNA damage as well as mediating a DNA repair mechanism (Zou and Elledge 2003). Replication stress and DNA damage is recognized by ataxia-telangiectasia mutated and Rad3-related (ATR)—ATR-interacting protein (ATRIP) complex. Studies have shown that this complex is specifically recruited by RPA to the sites of DNA damage and requires RPA for the complex association with ssDNA (Zou and Elledge 2003). Curiously, E. coli SSB is unable to recruit ATRIP to DNA, arguing for specific interactions of human RPA and ATRIP.

An analogous function of RPA was observed in somatic hypermutation (SHM) and class switch recombination, mediated through the action of activation-induced cytidine deaminase (AID). For a number of years the mechanism of AID's targeting to the sites with SHM motifs was not clear. Last year an elegant study revealed that recruitment of AID is mediated by RPA (Chaudhuri et al. 2004). Curiously, the interaction of AID with RPA was mediated through RPA32C.

The carboxyl-terminal domain of RPA was also implicated in interaction with a number of repair proteins (Mer et al. 2000b). Structural analysis of RPA32C revealed the presence of winged helix-loop-helix fold, which is a domain implicated in protein-protein interactions. Several repair proteins, UNG2, XPA, and RAD52, were shown to specifically interact with carboxyl terminus of RPA. Of particular interest was an observation that repair proteins interacted with winged helix motif in a very similar mode, suggesting a universal mode of RPA interaction with replication and repair proteins.

The model can be extended further to include other single-strand DNA binding factors. Analysis of Pot1 protein that binds ssDNA comprising telomeres was shown to regulate telomerase activity in budding yeast and human cells (Smogorzewska and de Lange 2004). Interestingly, Pot1 negatively regulates telomerase activity in vitro when telomeres are coated with Pot1 due to inability of telomerase to get access to DNA (Kelleher et al. 2005). However, in vivo Pot1 acts as a positive regulator of telomerase (Colgin et al. 2003). It is reasonable to speculate that the discrepancy between in vivo and in vitro scenario can easily be explained by an action of an accessory protein that would remove Pot1 from telomere to allow telomerase access. The emerging mechanism is very

similar to the interaction of RPA, Tag, and pol-prim in a viral replication model. The striking similarity between RPA and Pot1 apparent action provides strong support for universal mode of action for ssDNA binding proteins: coating ssDNA to prevent degradation by cellular nucleases and serving as a landing pad for enzymatic factors. The identity of a factor that would remove Pot1 from DNA and facilitate telomerase action is not clear, but Pot1 was shown to interact with several telomere-maintenance factors. Several candidates could include PIP1 and PTOP. Both proteins interact with Pot1 and assist in its recruitment to telomeres (Liu et al. 2004; Ye et al. 2004). It is also possible that the actions of more than one protein would be necessary to remove Pot1 from ssDNA.

Abundance of RPA *in vivo* guarantees that emerging ssDNA is immediately coated with RPA to prevent strand re-annealing and nuclease degradation. However, single-stranded DNA cannot exist in such a state for extended periods of time in a cell since persistent RPA-ssDNA or E. coli SSB-ssDNA will signal DNA damage, indicating that ssDNA "hand off" to the next replication factor failed. The presence of RPA serves as a signal for appropriate cellular factors to bind DNA and elicit their action. It is still unclear how RPA differentiates recruitment of replication, repair, or recombination factors. We can speculate that presence of additional determinants--other DNA and damage recognition proteins or dsDNA—can serve as fine-tune signals to specify the type of action necessary. Alternatively, a particular default algorithm of primosome assembly and hand off might exist and only if it fails, repair factors are recruited to ssDNA.

These data, combined with findings of this work, provide a basis to speculate that the carboxyl-terminus of RPA serves a function of a molecular "landing pad" for specialized repair or recombination complexes.

**RPA as a landing pad in cellular replication**

*In vitro* models can provide considerable insight into the mechanism and nature of inter-primosome interactions. Viral models can offer some *in vivo* interaction in transformed cells as in SV40 case and a great advantage of these systems lies in the fact that one or more viral proteins mediate viral genome replication. Therefore, use of viral proteins, such as Tag, allows to bypass multiple interactions required *in vivo* and focus on the interactions of interest. In addition, we can speculate on the nature of in vivo interactions among replication factors, based on the interactions observed in viral replication system.

The main question that still remains is whether the mechanisms revealed in studies of viral replication apply to the players of eukaryotic replication and if so how do they operate? The initiation of DNA replication begins when ORC hexamer assembles onto the replication origins (Bell 2002). The ORC complex is then required for Cdc6 recruitment to the origin. Recent data indicate that Cdc6 presence on chromatin is required for Cdt1 association with the complex (Tsuyama et al. 2005). Cooperative action of Cdc6 and Cdt1 is necessary for the recruitment of MCM2-7 complex. The function of MCM is not completely understood, but it is required for further steps of DNA replication. Since MCM2-7 sub-complexes, such as 4,6,7 exhibited weak helicase activity, we can speculate that it can be involved in non-processive dsDNA unwinding and upon fulfilling its action, is replaced by a processive helicase MCM8 (Maiorano et

al., 2005). In the SV40 system all the steps of early initiation are mediated and accomplished by Tag. When first ssDNA is generated, probably by MCM2-7, it is immediately occupied by RPA to prevent strand re-annealing. Data from the Fanning lab suggests that RPA is being actively loaded onto ssDNA through interactions with Tag. We can then envision a scenario in which MCM2-7 interactions with RPA are required for RPA association with chromatin. It is also possible that the replicative helicase MCM8 lacks this interaction and therefore MCM2-7 action is required for initiation of DNA replication. Since polymerase switch is mediated by RPA we can also assume its action in the helicase switch.

Data obtained from SV40 in vitro replication system, combined with structural analysis allowed us to gain considerable insights into primosome assembly and function. Though a lot is still unclear about DNA metabolism, emerging mechanism suggests major regulatory role performed by single-strand binding proteins in DNA replication, repair, somatic hypermutation, chromosome recombination, and telomere maintenance.

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