MSSP, a protein binding to an origin of replication in the c-*myc* gene, interacts with a catalytic subunit of DNA polymerase α and stimulates its polymerase activity

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Received 2 May 2000; received in revised form 23 May 2000

Edited by Ned Mantei

Abstract MSSP has been identified as a protein that binds to both single- and double-stranded sequences of a putative DNA replication origin sequence in the human c-*myc* gene. MSSP possesses versatile functions, including stimulation of DNA replication, transcriptional regulation, apoptosis induction, and cell transformation coordinated by c-Myc. MSSP contains two RNP domains, RNP1-A and RNP1-B, both of which are necessary for all of the functions of MSSP. In this study, we found that MSSP binds to the N-terminal region of a catalytic subunit of a human DNA polymerase α via its RNP domains both in vitro and in human cells. Furthermore, MSSP was released from the putative DNA replication origin of the c-*myc* gene after it complexed with DNA polymerase α, and MSSP stimulated DNA polymerase activity in vitro.

Key words: MSSP; DNA polymerase α; c-*myc*

1. Introduction

Initiation of DNA replication in eukaryotic cells occurs once per cell cycle, and mechanisms for achieving this regulation have been proposed on the basis of results in the yeast system. Generally, a protein complex termed ORC first recognizes the origin of DNA replication in the genome (*ori*), and then CDC6 binds to the ORC–*ori* complex. After MCM proteins are recruited by CDC6 to the ORC–*ori* complex, S-phase-specific protein kinases, including CDK2 and CDC7, modify MCM to recruit DNA polymerases that start DNA replication (see reviews [1–4]). Although the precise structures and locations of *ori* in mammalian cells are not well understood, more than 10^5 *ori*s are thought to exist in mammalian cells. It is therefore reasonable to speculate that in addition to the basal replication factors such as ORC, CDC6 or MCM, specific factors exist to specify a particular *ori* in the genome by association with the basal replication factors. We previously identified a putative *ori* in the human c-*myc* gene, myc(H-P), which works as an autonomous replicating sequence in human cells [5,6]. The protein termed MSSP has been shown to bind to both single- and double-stranded myc(H-P) sequences [7], and two cDNAs encoding MSSP-1 and MSSP-2, a splicing variant of MSSP-1, have been cloned [7–9]. MSSP-1 and MSSP-2 possess two RNP consensus sequences, RNP-1A and RNP-1B, and both RNPs are necessary for DNA binding [8]. The different biological functions of MSSP-1 and MSSP-2 have not yet been identified. MSSP stimulates a modified origin of SV40 DNA replication, in which the TATA sequence in the original SV40 sequence is replaced by an MSSP recognition sequence [7,10], and works as a transcription repressor in the α-smooth muscle actin gene [11]. Furthermore, MSSP stimulates c-Myc-derived apoptosis induction [13] and cell transformation by binding to c-Myc [12]. This transformation-stimulating activity is parallel to the DNA replication activity of MSSP [13]. Preliminary results also show that MSSP interacts with MCM (data not shown). These results suggest that MSSP plays a role as a DNA replication factor in the initiation of DNA replication in the human c-*myc* gene. In this study, we found that MSSP interacts with a catalytic subunit of DNA polymerase α in vitro, and with a DNA polymerase α complex containing the catalytic subunit in cultured cells, and that MSSP stimulates DNA replication activity of DNA polymerase α.

2. Materials and methods

2.1. Cells

Human KB cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

2.2. Plasmids

The plasmids containing segments of DNA polymerase α cDNA were kindly provided by Dr. Teresa S.-F. Wang. All of the plasmids used in this study were constructed by the insertion of PCR-based amplified fragments into the respective vectors, and some of the plasmids were reported previously [13]. Details of the construction procedures of the plasmids are available upon request.

2.3. In vitro and in vivo binding assays

GST–MSSP-1, GST–MSSP-2, GST-deletion mutants of polymerase α, and GST were purified from *Escherichia coli* BL21(DE3) transformed with pGEX-MSSP-1, pGEX-MSSP-2, pGEX-polα-A, pGEX-polα-B, pGEX-polα-C, and pGEX-6P-1 as described previously [14]. GST-free MSSP-1 and MSSP-2 were prepared after digestion of GST–MSSP-1 and GST–MSSP-2 with PreScission protease according to the supplier's manual (Amersham Pharmacia). 1 μg of the purified GST–polα-A, GST–polα-B, GST–polα-C, or GST was first applied to a column of glutathione Sepharose 4B (Amersham Pharmacia) in a buffer containing 50 mM Tris–HCl (pH 7.5), 100

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0014-5793/00/$20.00
PII: S0014-5793(00)01679-3

mM NaCl, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml bovine serum albumin. Then 1 μg GST-free MSSP was applied to the column. After extensive washing of the column with the same buffer as described above, the proteins bound to the resin were recovered, separated in a 10% polyacrylamide gel containing SDS, blotted against an anti-MSSP antibody [13], and visualized by the ECL system (Amersham Pharmacia). pGEM-MSSP-1, pBS-MSSP-2, and its deletion mutants were first digested with *Sca*I and used as a template for in vitro transcription with T7 RNA polymerase. RNA products were used for in vitro translation with rabbit reticulocyte lysate (Promega). ^{35}S-labeled MSSPs and 1 μg of the purified GST–polα-A, GST–polα-B, GST–polα-C or GST that had been trapped by the glutathione Sepharose 4B were mixed, and then treated by the same procedure as described above. After extensive washing of the resin with a washing buffer, the bound proteins were boiled in Laemmli buffer, separated in a 10% polyacrylamide gel containing SDS, and fluorographed. Co-immunoprecipitation was performed using human KB cell lysates in a lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 0.2% NP-40, 2 mM EDTA, and 2 mM dithiothreitol). After the cell extracts were immunoprecipitated with an anti-polymerase α- or an anti-EE-antibody-conjugated Sepharose for 30 min at 4°C and washed five times with the lysis buffer, the bound proteins were separated in a 10% polyacrylamide gel and detected as described above.

2.4. Electrophoretic mobility shift assay

The binding reactions were carried out using the radiolabeled minus strand c-*myc ori* sequence (5′-GATCCACTATTCAACCGCATAAGAGAG-3′) as described previously [7]. The resulting protein–DNA complexes were resolved on 4% polyacrylamide gels containing 0.25×TBE (22.5 mM Tris, 22.5 mM H_3BO_4, and 0.63 mM EDTA).

2.5. DNA polymerase α activity

DNA polymerase α assay was performed according to the methods of the T.S.-F. Wang laboratory as described previously [15]. Briefly, 0.058 μg (0.3 pmol) of a catalytic subunit of DNA polymerase α purified from SF9 cells infected with a baculovirus encoding DNA polymerase α was mixed with various amounts of thioredoxin-tagged MSSP purified from *E. coli* cells in a mixture containing 25 mM Tris (pH. 7.5), 10% glycerol, 50 mM NaCl, 0.1 mM EDTA, [^{32}P]dATP, and activated calf thymus DNA as a template, and these were incubated at 37°C for 15 min. Then acid-insoluble radioactivity was measured.

3. Results and discussion

3.1. Interaction of MSSP with DNA polymerase α in vivo

Since MSSP stimulates DNA replication of the SV40-modified plasmid in which the TATA sequence of SV40 DNA has been replaced by an MSSP recognition sequence of the c-*myc* gene [7,10], we examined the interaction between MSSP and DNA polymerase α under physiological conditions. A whole cell extract of human KB cells was immunoprecipitated with an anti-DNA polymerase α antibody or non-specific EE antibody, and the precipitate was blotted using an anti-MSSP antibody (Fig. 1). MSSP was detected in the precipitate with the anti-DNA polymerase α antibody but not in the precipitate with the non-specific EE antibody (Fig. 1A, lanes 1 and 2). Although the anti-MSSP-1 antibody used here recognizes MSSP-2 as well as MSSP-1, the two proteins were not discriminated under these conditions, due to the small difference of the molecular weights between the two proteins. These results clearly indicate that MSSP is associated with DNA polymerase α in cells.

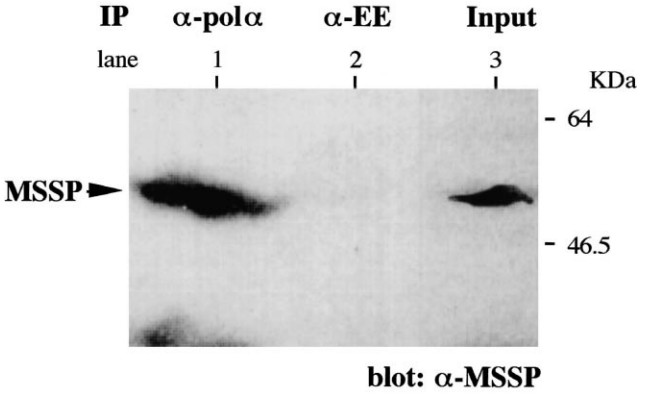

Fig. 1. Association of MSSP with DNA polymerase α in vivo. The extracts of KB cells were prepared and subjected to an immunoprecipitation reaction using an anti-DNA polymerase α or non-specific EE antibody (lanes 1 and 2, respectively). The precipitates were blotted with an anti-MSSP polyclonal antibody. A 1/20 amount of the cell extract used for the reactions (lane 3, Input) was analyzed in parallel.

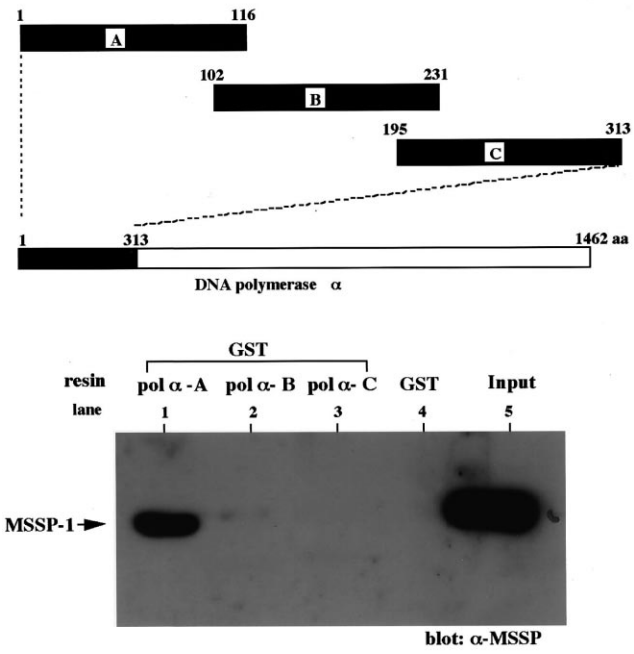

Fig. 2. In vitro binding activity of MSSP-1 to DNA polymerase α GST fusion proteins of the three N-proximal regions of DNA polymerase α, A, B and C, was expressed in *E. coli* and purified. GST–MSSP-1 was also prepared as described above and digested with PreScission protease to yield GST-free MSSP-1. The GST fusion proteins or GST alone were applied to the glutathione beads and incubated with MSSP-1. After extensive washing, the proteins that had bound to the beads were analyzed in an SDS-containing polyacrylamide gel (10%) and blotted with an anti-MSSP antibody. One-twentieth volumes of the MSSP-1 used for the binding reaction were applied to the same gel (lane 5).

3.2. Binding of MSSP with DNA polymerase α in vitro

To identify the domains of DNA polymerase α that interact with MSSP-1, we first used a yeast two-hybrid assay. The cDNA segments of DNA polymerase α used for identification of domains interacting with SV40 T antigen [15] were kindly supplied by Dr. T.S.-F. Wang, and these were inserted in frame downstream of the GAL4 DNA binding domain in the pAS2 vector. Each of these plasmids was co-transformed with pGAD-MSSP-1 containing the GAL4 activation domain fused to MSSP-1 into *Saccharomyces cerevisiae* Hf7C, and β-galactosidase activity of the resultant colony was examined. The results showed that the N-terminal fragment of DNA polymerase α spanning amino acids 1–116 gave a blue colony

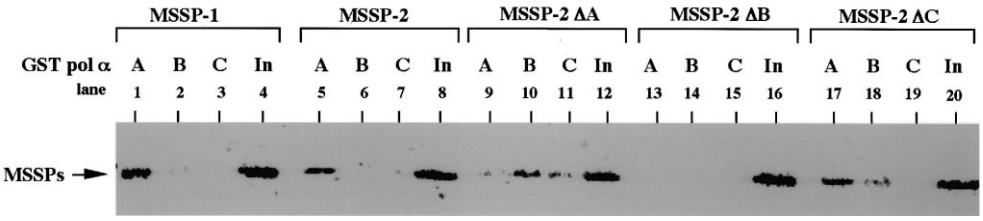

Fig. 3. Determination of the binding region of MSSP with DNA polymerase α. GST fusion proteins of the three N-proximal region of DNA polymerase α, A, B and C, were applied to the glutathione beads and incubated with in vitro translated ^{35}S-labeled MSSP-1, MSSP-2 or its deletion mutants. After extensive washing, the proteins that had bound to the beads were analyzed in an SDS-containing polyacrylamide gel (10%) and subjected to fluorography. One-twentieth volumes of the MSSP-1 used for the binding reaction were applied to the same gel (In, lanes 4, 8, 12, 16 and 20).

indicative of positive β-galactosidase activity (data not shown). We then examined the direct interaction between MSSP-1 and DNA polymerase α. The three segments of DNA polymerase α, polα-A spanning amino acids 1–116, polα-B spanning amino acids 102–231 and polα-C spanning amino acids 195–313, were expressed as fusion proteins with glutathione *S*-transferase (GST) and affinity-purified by glutathione Sepharose resin. Full-size MSSP-1 was first expressed as a fusion protein of GST, and MSSP-1 was prepared after cleaving off GST by PreScission protease. Then an in vitro binding assay, the so-called pull-down assay, was carried out by incubating polαs and MSSP-1 on glutathione Sepharose resin, and the bound protein(s) detected by Western blotting probed with an anti-MSSP antibody (Fig. 2). As was suggested by the results of the yeast two-hybrid assay, the results showed that MSSP-1 directly bound to polα-A but not to polα-B, polα-C or GST (Fig. 2, lanes 1–4).

MSSP family proteins contain two RNP-1 consensus motifs, which are essential for the specific DNA binding of the proteins [8]. To determine the DNA polymerase α binding region within MSSP, MSSP-1, MSSP-2 or deletion mutants of MSSP-2 translated in vitro in the presence of [^{35}S]methionine were mixed with GST–polα-A, GST–polα-B, GST–polα-C or GST, and pull-down assay was carried out. Deletion of the first RNP-1 motif, RNP-1A, corresponding to amino acids 70–77 of MSSP-2 (mutant MSSP-2ΔA), or the second RNP-1 consensus motif, RNP-1B, corresponding to amino acids 149–156 of MSSP-2 (mutant MSSP-2ΔB), abolished the specific binding activity (Fig. 3, lanes 9–16). M2ΔA showed a weak non-specific binding to polα-B and polα-C (Fig. 3, lanes 9–12). Deletion of C-terminal amino acids 328–337 (mutant MSSP-2ΔC), on the other hand, gave rise to strong binding to polα-A and weak binding to polα-B, but no binding to polα-C (Fig. 3, lanes 17–20). Both MSSP-1 and MSSP-2 were thus shown to bind to DNA polymerase α, and the two RNP-1 motifs of the MSSP proteins were thought to be essential for the specific binding.

3.3. Abrogation of myc(H-P) binding activity of MSSP by DNA polymerase α

Since MSSP binds to both double- and single-stranded myc(H-P) sequences and MSSP binds to the N-terminal region of DNA polymerase α, polα-A, the effect of polα-A on the DNA binding activity of MSSP-1 to a minus strand of myc(H-P) was examined by a mobility shift (band shift) assay (Fig. 4). GST–MSSP-1, GST–polα-A and GST–polα-C were first purified from *E. coli* cells, and GST was cleaved off by PreScission protease. Then various amounts of recombinant polα-A or polα-C and MSSP-1 were incubated with labeled oligonucleotides corresponding to the minus strand myc(H-P) as a probe. No nucleoprotein complex was observed in the incubation of the probe with polα-A or polα-C by itself (Fig. 4, lanes 6 and 12). While the formation of an MSSP–myc(H-P) complex was not affected by the addition of any amounts of polα-C (Fig. 4, lanes 8–11), the MSSP-1–myc(H-P) complex, on the other hand, was decreased by the addition of polα-A in a dose-dependent manner (Fig. 4, lanes 2–5). The results suggest that polα-A–MSSP-1 complex loses the ability to bind to the myc(H-P) sequence.

3.4. Stimulation of DNA polymerase α activity by MSSP

MSSP-1 tagged with both thioredoxin (Trx) at the N-terminus and 6× histidine at the C-terminus was expressed in *E. coli* and purified by a nickel-affinity resin. Various amounts of Trx–MSSP-1 were mixed with the purified DNA polymerase α from Sf9 cells infected with a baculovirus encoding DNA polymerase α [15], and polymerase activity was examined on activated calf thymus DNA as a template (Fig. 5). Trx–

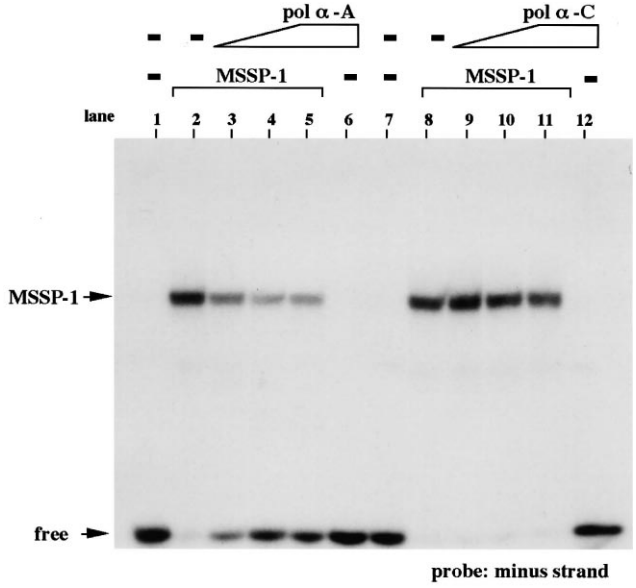

Fig. 4. Abrogation of the DNA binding activity of MSSP-1 by DNA polymerase α. 2.5 ng of purified MSSP-1 and 250 (lanes 3 and 9), 500 (lanes 4 and 10) and 1000 ng (lanes 5 and 11) of the A or C fragment of DNA polymerase α were reacted with ^{32}P-labeled single-strand myc(H-P) oligonucleotides, and bandshift assay was carried out as described in Section 2. The DNA–protein complex was separated in 4% native polyacrylamide gel.

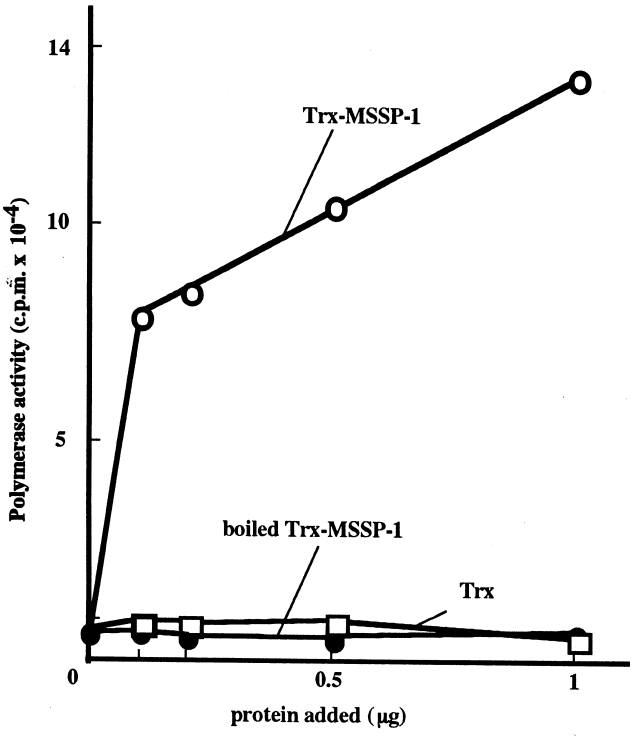

Fig. 5. Stimulation of polymerase α activity by MSSP-1. 1 μg of the purified DNA polymerase α and various amounts of thioredoxin-tagged MSSP-1 (Trx–MSSP-1), boiled Trx–MSSP-1 or Trx were mixed, and a polymerase assay was carried out as described in Section 2. After the reaction, the acid-insoluble radioactivity was measured.

MSSP-1 stimulated the activity of DNA polymerase α in a dose-dependent manner, while neither the boiled Trx–MSSP nor Trx alone did. These results suggest that MSSP is a modulator of DNA polymerase α.

In this study, we found that (1) MSSP interacts with a catalytic subunit of DNA polymerase α in vitro and with DNA polymerase α complex containing the catalytic subunit in vivo, and that both RNP domains of MSSP are necessary for binding, (2) this complex loses the ability to bind to the myc(H-P) sequence, and (3) MSSP-1 stimulates polymerase activity of DNA polymerase α. RNP domains of MSSP are essential for binding to both c-Myc and the myc(H-P) sequence [7], and for biological functions of MSSP, including stimulation of DNA replication [7,8], apoptosis induction [12], and cell transformation [13]. It is therefore speculated that abrogation of DNA binding activity of MSSP by DNA polymerase α is due to a competition between polymerase α and DNA for binding to the RNP in MSSP. The results of this study and those of another study showing that MSSP stimulates DNA replication of a plasmid containing the myc(H-P) sequence in human cells [7,8] suggest that MSSP is a modulator of DNA replication. The character of MSSP is reminiscent of SV40 T antigen, an initiation protein encoded by SV40. In initiation of SV40 DNA replication, T antigen binds to the *ori* of SV40 DNA and then binds to the N-proximal region of DNA polymerase α, polα-C, to start replication [15]. Since an AT-rich sequence is present beside the myc(H-P) sequence in the human c-*myc* gene, the mechanism of initiation of DNA replication in this c-*myc* region is speculated to be as follows. An ORC complex might recognize this AT-rich sequence to define the potential *ori* region and then a pre-replication complex might be formed. MSSP may be involved in this complex to specify the myc(H-P) sequence. After DNA polymerase α is recruited by the pre-replication complex, MSSP binds to polymerase α to start replication by stimulating the polymerase activity. Our preliminary data show that MSSP binds to an MCM subunit (data not shown). To prove this hypothesis, further analysis of the interaction between MSSP and components of the pre-replication complex is needed.

Acknowledgements: We are grateful to Dr. T.S.-F. Wang for providing several materials and the place for conducting the initial experiment of this study. We thank Kiyomi Takaya and Yoko Misawa for their skillful technical assistance. This work was supported by grants-in-aid from the Ministry of Education, Science, Culture and Sport of Japan.

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