Interactions between p53, hMSH2–hMSH6 and HMG I(Y) on Holliday junctions and bulged bases

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Received February 6, 2002; Revised and Accepted April 9, 2002

ABSTRACT

The ability of the tumor suppressor protein, p53, to recognize certain types of DNA lesions may represent one of the mechanisms by which this protein modulates cellular response to DNA damage. p53 DNA binding properties are regulated by several factors, such as post-translational modifications including phosphorylation and acetylation, regulation by its own C-terminal domain and interactions with other cellular proteins. Substrates resembling Holliday junctions and extra base bulges were used to study the effect of three nuclear proteins, HMG-1, HMG I(Y) and hMSH2–hMSH6, on the lesion binding properties of p53. Gel retardation assays revealed that the three proteins had varying effects on p53 binding to these substrates. HMG-1 did not influence p53 binding to Holliday junctions or 3-cytosine bulges. HMG I(Y) rapidly dissociated p53 complexes with Holliday junctions but not 3-cytosine bulges. Finally, the mismatch repair protein complex, hMSH2–hMSH6, enhanced p53 binding to both substrates by 3–4-fold. Together, these results demonstrate that p53 DNA binding activity is highly influenced by the presence of other proteins, some having a dominant effect while others have a negative effect.

INTRODUCTION

p53 is a key cell cycle checkpoint factor which causes cells to undergo either cell cycle arrest or apoptosis following DNA damage. When cells are exposed to UV and X irradiation, there is an accumulation and activation of p53 (reviewed in 1–3) which results in the downstream transactivation of several genes whose products assist in the maintenance of genomic integrity (4,5). However, the exact mechanism of p53 activation following DNA damage is not clear. Post-translational modifications such as phosphorylation and acetylation appear to play an important role in this process (6,7). Additionally, the transcriptional response of p53 requires tetramerization and this could be influenced by several factors (8). Finally, p53 activity could be altered by interactions with other proteins. Indeed, p53 has been shown to associate *in vitro* and *in vivo* with a number of proteins involved in DNA metabolic

processes such as repair [XPB, XPD (9) and WRN (10)], replication [DNA polymerase (11) and RPA (12)] and recombination [hRad51 (13)].

Although the p53 response to DNA damage is largely mediated by downstream proteins, there is increasing evidence that p53 can directly interact with DNA lesions. p53 can bind single strand and double strand breaks (14), three-stranded DNA structures (15,16), extra base bulges (17) and several base/base mismatches in DNA (18). Additionally, we have shown that p53 binds with extremely high affinity to Holliday junctions (16). All of these lesions occur as a result of typical recombination, replication and repair processes as well as DNA damage caused by environmental factors. For example, Holliday junctions are natural intermediates of homologous recombination; however, high levels of sister chromatid exchange following irradiation suggests the formation of these structures due to DNA damage (19). Replication of molecules containing thymidine dimers could result in the insertion of additional nucleotides opposite the existing lesion. Additionally, when two similar DNA molecules with a few non-identical bases undergo recombination, regions of extra base bulges and mismatches can be left behind. Replication errors can also lead to the generation of mismatches and extra base bulges in DNA. All of these lesions need to be repaired for cell cycle progression to occur. The high affinity of p53 to several of these lesions strongly points to an important role in damage recognition as well as downstream repair and recombination pathways. Thus, p53 response to lesions in DNA caused by normal metabolic processes and DNA damaging agents may be 2-fold: first, to transactivate downstream genes that enable cell cycle arrest or apoptosis and, secondly, to recognize and bind these lesions to signal repair pathways.

The importance of p53 DNA binding (both sequence dependent for transactivation and sequence independent for lesion recognition) suggests that this activity is tightly regulated by a number of factors to ensure proper p53 function at the correct times during the cell cycle. p53 DNA binding activity is affected by post-translational modifications such as phosphorylation, acetylation and sumolation (reviewed in 20). Binding by the monoclonal antibody, pAB421 (21,22), as well as interaction with single strand DNA (23) activates sequence-specific DNA binding. The identification of several cellular and viral proteins that associate with p53 suggests that protein–protein interactions play an important role in the regulation of its DNA binding activity. This is further supported by the demonstration that Ref-1 is a potent activator

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of p53 DNA binding activity (24). Additionally, Jayaraman *et al.* (25) have shown that the high mobility group (HMG) protein HMG-1 stimulates sequence-specific p53 DNA binding.

The HMG proteins are among the largest group of non-histone chromatin proteins. HMG proteins can be classified into three groups: the HMG-1/2, HMG I(Y) and HMG-14/17 families (reviewed in 26). The exact cellular functions of these proteins are not fully understood. They are architectural elements that bind unusual structures in DNA and have low sequence specificity. The HMG-1/2 family is the most abundant of this group of proteins. They interact with DNA through two conserved DNA binding domains known as the HMG boxes (27,28) and preferentially bind DNA structures that contain sharp angles such as cruciforms, four-way junctions (29) as well as cisplatin-DNA adducts (30,31). The HMG I(Y) proteins contain A-T hook domains that serve as DNA binding motifs and can recognize four-way junctions (32). Both groups of proteins can induce bends in linear DNA templates as well as introduce supercoils in topologically constrained molecules (33). There is growing evidence that these proteins may represent a new class of chaperone factors that can facilitate the interactions of other proteins with their respective target sequence. For example, HMG-1 induces a structural change in the target sequence of the progesterone receptor, thus facilitating protein binding (34). Additionally, HMG I(Y) has been shown to regulate long range enhancer-dependent transcription by altering DNA topology (35). Finally, as mentioned above, HMG-1 can enhance sequence-specific binding of p53 (25). Thus, this group of proteins seems to enable the 'loading' of other proteins on their target sequences. The ability of the HMG group proteins to recognize unusual DNA structures and their influence on various protein-DNA interactions, as well as the fact that HMG-1 stimulates the sequence-specific binding of p53, makes them good candidates for factors that might affect the lesion binding properties of p53. Thus, it was critical to ask whether any of these HMG proteins influences p53 recognition of DNA damage. In this study, the effects of two HMG group proteins, HMG-1 and HMG I(Y), on p53 binding to DNA lesions were examined. Two different substrates, one containing three 3-cytosine bulges and the other resembling Holliday junctions, were used to monitor the effect of these HMG proteins on p53 DNA binding activity by gel retardation assays.

Lesions such as mismatches and extra base bulges are recognized by the *Escherichia coli* mismatch repair protein, MutS, and its eukaryotic counterparts (MSH2, MSH3 and MSH6) (36 and references therein). p53 and hMSH2-hMSH6 share a number of common features. Both can recognize extra base bulges and Holliday junctions *in vitro* (16,17,37,38). Both proteins can inhibit DNA recombination when they encounter DNA lesions both *in vivo* and *in vitro* (39,40). Additionally, these proteins play an important role in genome maintenance by interacting with other repair and replication factors. The relevance of p53 lesion recognition events within the cell was demonstrated by comparing the effect of the HMG proteins on the DNA binding activity of hMSH2-hMSH6. Our results indicate that HMG I(Y) can effectively compete with p53 and hMSH2-hMSH6 for Holliday junction binding. However, HMG I(Y) had no effect on p53 binding to 3-cytosine bulges while dissociating hMSH2-hMSH6 complexes with the same substrate. On the other hand, it was found that hMSH2-hMSH6

had a stimulatory effect on p53 binding to both Holliday junctions and 3-cytosine bulges. Thus, p53 lesion binding activity can be modulated in different ways—negative regulation is seen with HMG I(Y) and positive with hMSH2-hMSH6.

MATERIALS AND METHODS

DNA probes

Four-way junction probes were prepared by annealing four oligonucleotides, K1, K2, K3 and K4 (synthesized and HPLC purified by Gibco-BRL Inc.) to produce J12 junctions as described previously (16). Briefly, oligonucleotide K1 was end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase (NEB Inc.) followed by the addition of equal amounts of the other three oligonucleotides (unlabeled). The mixture was heated at 65°C for 10 min in the presence of 0.4 M NaCl and allowed to slowly cool to room temperature for 6–24 h. A duplex control probe was prepared by annealing an oligonucleotide containing the complementary sequence to the labeled K1 strand. The annealed products were purified on 10% non-denaturing polyacrylamide gels. The probe containing 3-cytosine bulges were prepared by annealing a 79 nt oligonucleotide (5'-AGT CGG AAT TCG GCT CGA **CCC** AGG **CCC** CGT **CCC** TCT CGG AGC ACT GCA GAA CCG CTT TGG CCG CCG CCG GAT CCC GAG T-3') to a 70 nt oligonucleotide (5'-ACT CGG GAT CCG GCG GCG GCC AAA GCG GTT CTG CAG TCG TCC GAG AAC GGG TTC GAG CCG AAT TCC CAG T-3'). This results in a duplex probe, which has three sets of 3-cytosine bulges on the top strand (indicated in bold). As described above, the top strand was first end-labeled, followed by annealing to the bottom strand and purification on a 10% polyacrylamide gel.

Proteins

Human p53 was overexpressed in insect SF9 cells using a baculovirus vector provided by Dr Arnold Levine and purified as described previously (41). The hMSH2-hMSH6 heterodimer was purified as described previously from baculovirus infected cells using a vector provided by Dr Richard Fishel (42).

HMG1 was purified from calf thymus tissue as described previously (43). Approximately 0.7 mg of HMG-1 (molecular weight 28 kDa) was recovered from 65 g of calf thymus tissue and the protein was found to be protease and nuclease free. The protein was purified to 95% homogeneity as determined by SDS-PAGE analysis and was reactive to an anti-HMG-1 antibody but not an anti-HMG-2 antibody on western blots (data not shown).

HMG I(Y) was overexpressed in *E.coli* and purified as described previously (44) using a vector provided by Dr Michael Bustin. Approximately 0.8 mg of HMG I(Y) (molecular weight 22 kDa) was obtained from 1.5 l of bacterial cells and the protein (95% homogeneity as determined by SDS-PAGE) was found to be free of nuclease and protease activity.

Gel retardation assays

Reactions (20 μ l) containing probes (7.5 nM) and the proteins (see figure legends for concentrations) were incubated at room temperature for 20 min in a buffer containing 10 mM HEPES-KOH pH 7.8, 25 mM KCl, 0.5 mM DTT, 0.01 mM

EDTA, 10% glycerol and 50 ng of salmon sperm DNA as non-specific competitor. The reactions were adjusted to 10% glycerol and loaded on 5% non-denaturing polyacrylamide gels in 0.5× TBE (45 mM Tris–borate, 1 mM EDTA). The gels were run at 200 V for 2.5 h at 4°C, dried and analyzed by autoradiography and quantified using a Storm 840 phosphorimager (Molecular Dynamics).

RESULTS

HMG-1 does not stimulate p53 binding to Holliday junctions

Templates that resemble Holliday junctions were prepared by annealing four oligonucleotides based on the J12 molecule, the properties of which have been described previously (45). This creates a four-way junction with the crossover point centrally located that is capable of branch migrating over a 12 bp core. These junctions have been used extensively as model junctions for binding studies with p53, yMSH2 and yMSH2–yMSH6 (16,38,46). Gel retardation assays were performed to determine whether HMG-1 influences p53 binding to Holliday junctions. p53 and HMG-1 were incubated with the probes at room temperature for 20 min followed by separation of the protein/DNA complexes from free DNA by non-denaturing polyacrylamide gel electrophoresis. Molar ratios of 1:10 and 1:15 of DNA to p53 (tetramers) were used and increasing amounts of HMG-1 were added to achieve a p53 to HMG-1 molar ratio that ranged from 1:0.1 to 1:2. Duplex DNA containing one of the strands of the J12 junction and a complementary sequence for the lower strand was used as a negative control. As observed previously, p53 has high affinity for the four-way junctions (Fig. 1, lanes 2 and 8) but does not bind to the duplex control (lane 16) indicating the specificity of this protein to four-way structures. When increasing amounts of HMG-1 were added to the p53 containing reactions, there was no visible change in the levels of the p53/Holliday junction complexes (lanes 3–7 and 9–13). Even a 10-fold excess of HMG-1 over p53 did not elicit an increase in p53 binding to Holliday junctions (data not shown). The activity of HMG-1 was confirmed by examining its ability to enhance p53 sequence-specific binding. In concurrence with the data of Jayaraman *et al.* (25), p53 binding to its consensus sequence was stimulated by the addition of purified HMG-1 at the same concentrations used with the Holliday junction templates (data not shown). These results indicate that the HMG-1 we purified is active but does not enhance p53 binding to Holliday junctions. While HMG-1 is involved in promoting p53 binding to its consensus sequence, another member of this family of proteins may be responsible for regulating its binding to Holliday junctions and lesions in DNA.

HMG I(Y) causes dissociation of p53 from Holliday junction substrates

The HMG I(Y) class of proteins has been shown to have a different spectrum of activities from HMG-1 and to have a much higher affinity for four-way junctions (47); thus, these proteins seemed more likely to influence the binding of p53 to Holliday junction molecules. Gel retardation assays were performed using the same conditions as the HMG-1 reactions. p53 was added at a 1:10 ratio of DNA to tetramers and the molar ratio

Figure 1. HMG-1 does not significantly affect p53 binding to Holliday junctions. Holliday junction probes (7.5 nM) were incubated with p53, HMG I(Y) or both proteins at room temperature for 20 min. The reaction products were separated by non-denaturing polyacrylamide gel electrophoresis followed by autoradiography. Lanes 1 and 15, probe; lanes 2–7 and 16, 75 nM p53; lanes 8–13, 115 nM p53, lanes 3 and 9, 10 nM HMG1; lanes 4 and 10, 20 nM HMG1; lanes 5 and 11, 40 nM HMG1; lanes 6 and 12, 80 nM HMG1; lanes 7 and 13, 170 nM HMG1; lanes 14 and 17, 17 nM HMG1. Arrows and brackets indicate the positions of the probe, p53/DNA complexes and HMG1/DNA complexes.

of p53 to HMG I(Y) ranged from 1:0.1 to 1:2. As shown in Figure 2A, in the absence of HMG I(Y), p53 bound with high affinity to Holliday junction probes (lane 2). HMG I(Y) also bound to Holliday junction probes (lane 8) as well as the duplex probe (lane 14) albeit at a lower level than the four-way junctions. HMG I(Y)/Holliday junction complexes have a higher mobility than the p53 complexes, thus allowing clear distinction between the two protein–DNA species. When p53 and HMG I(Y) were incubated together with the Holliday junction probe, there was a decrease in the specific p53/DNA complexes as increasing amounts of HMG I(Y) were added (lanes 3–7). Addition of 20 nM HMG I(Y) caused an almost complete elimination of the p53/Holliday junction complexes. As seen in Figure 2A, multiple HMG I(Y)/DNA complexes were detected when higher amounts of this protein were added (lanes 7 and 8). It is likely that at this concentration, the template is saturated by HMG I(Y) making it unavailable for p53. However, when 10 nM HMG I(Y) was added (which represents a equimolar ratio of protein to DNA), unbound probe was seen on the gel, indicating that there was substrate available for binding, yet p53/DNA complexes were reduced by 20%. These results suggest an active dissociation of the p53/Holliday junction interactions by HMG I(Y).

To examine the mechanism by which HMG I(Y) eliminates p53 interactions with Holliday junctions, p53/DNA complexes were pre-formed by incubating the protein with the probe for 20 min at room temperature followed by the addition of HMG I(Y) for various amounts of time. If HMG I(Y) competes with p53 for binding sites, then the p53 bound Holliday junctions should be unavailable to HMG I(Y) and would continue to exist over time. However, if HMG I(Y) dissociates p53 from

Figure 2. HMG I(Y) effects on p53 complexes with Holliday junction and 3-cytosine bulge probes. (A) Dissociation of p53/Holliday junction complexes by HMG I(Y). Holliday junction and duplex probes (7.5 nM) were incubated with either p53, HMG I(Y) or both proteins at room temperature for 20 min. The reaction products were separated by non-denaturing polyacrylamide gel electrophoresis followed by autoradiography. The concentrations of proteins used are indicated above each lane. Arrows and brackets indicate the positions of the probe, p53/DNA complexes and HMG I(Y)/DNA complexes. (B) Pre-formed p53/Holliday junction complexes are also dissociated by HMG I(Y). Holliday junction probes (7.5 nM) were pre-incubated with p53 (115 nM) for 20 min followed by the addition of HMG I(Y) (20 nM) for increasing amounts of time. The reaction products were analyzed by non-denaturing polyacrylamide gel electrophoresis. (C) The rate of dissociation of p53/Holliday junction complexes is similar at three concentrations of p53 tested. Holliday junction probes were incubated with 34 nM (circles), 75 nM (squares) or 115 nM p53 (triangles) in the presence of increasing amounts of HMG I(Y) followed by gel retardation assays. p53/DNA complexes were quantified, normalized to complex formation in the absence of HMG I(Y), and plotted as a function of HMG I(Y) concentration.

the DNA, then these complexes would disappear over the time course. As seen in Figure 2B, pre-formed p53/Holliday junction interactions were eliminated within 2 min of HMG I(Y) addition. These results indicate that the HMG I(Y) is able to dissociate p53 actively from Holliday junctions.

To measure the rate of dissociation of the p53/DNA complexes, increasing amounts of HMG I(Y) were added to three different concentrations of p53 in gel retardation assays and the p53/DNA complexes were quantified. Binding in the absence of HMG I(Y) was considered as 100% and the fold reduction in p53/DNA complexes was determined and plotted as a function of HMG I(Y) concentration (Fig. 2C). Approximately 40 nM HMG I(Y) was sufficient to cause a 50% reduction of the p53/DNA complexes at all three concentrations of protein tested. This would correspond to 0.2 molecules of HMG I(Y) per p53 tetramer at the highest concentration of p53 tested.

Figure 3. Effect of HMG I(Y) on p53 binding to 3-cytosine bulge containing templates. (A) HMG I(Y) has no significant effect on p53 binding to DNA containing 3-cytosine bulges. The 3-cytosine bulge probe (7.5 nM) was incubated with 75 nM p53 and increasing amounts of HMG I(Y) and products analyzed by gel retardation assays. Protein concentrations are indicated above the lanes. (B) p53 complexes with the 3-cytosine bulge probe remain constant at all concentrations of HMG I(Y) tested. Probes containing 3-cytosine bulges (7.5 nM) were incubated with 75 nM (circles) or 115 nM p53 (squares) in the presence of increasing amounts of HMG I(Y) followed by gel retardation assays. p53/DNA complexes were quantified and plotted as a function of HMG I(Y) concentration.

HMG I(Y) does not affect the interaction of p53 with 3-cytosine bulges

We previously showed that p53 binds substrates containing single and multiple 3-cytosine bulges. The presence of three 3-cytosine bulges could result in a severe kink in duplex DNA and it has been shown that the HMG proteins tend to bind distortions in DNA (48). To determine whether HMG I(Y) has any effect on p53 binding to other recombination by-products, DNA molecules were designed such that they contain three 3-cytosine bulges separated by 3 bp on one of the strands (see Materials and Methods). Gel retardation assays were performed using the same ratios of p53 and HMG I(Y) as used with the Holliday junction probe (Fig. 3A). Complexes between p53 and the 3-cytosine bulge were detected in the absence of HMG I(Y) (lane 2). However, upon addition of HMG I(Y), these complexes remained either unchanged or showed a slight increase. The complexes were quantified, normalized to the value that was detected in the absence of HMG I(Y) and plotted as a function of HMG I(Y) concentration (Fig. 3B). These results show that HMG I(Y) did not significantly influence p53 interactions with the 3-cytosine bulge probe and complex formation remained fairly constant across the range

of HMG I(Y) tested. In fact, a slight increase was detected at some of the concentrations tested. HMG I(Y) can bind on its own to the 3-cytosine bulge probe (lane 3); however, since this probe is smaller than the Holliday junction template, these complexes do not effectively separate from the free probe. Therefore, it is likely that HMG I(Y) interacts with the 3-cytosine bulges in a manner that does not affect p53 binding to these substrates.

HMG I(Y) dissociates hMSH2–MSH6 complexes formed with both Holliday junctions and 3-cytosine bulges

Holliday junctions and 3-cytosine bulges are also recognized by the mismatch repair protein complex, hMSH2–hMSH6, which participates with other proteins to repair these lesions via the mismatch or recombination repair pathways. To determine whether HMG I(Y) affects hMSH2–hMSH6 binding activity, gel retardation assays were performed using Holliday junction probes and the two proteins. As seen in Figure 4A, hMSH2–hMSH6 (1:10 molar ratio of DNA to heterodimers) showed strong binding to the Holliday junction probes (lane 2). A low level of binding was also seen with the duplex control (lane 9) which is expected as hMSH2–hMSH6 has been shown to exhibit some non-specific DNA binding (42). Addition of increasing amounts of HMG I(Y) resulted in a decrease in the hMSH2–hMSH6/DNA complexes (Fig. 4A) and the dissociation curve of these complexes resembled that seen with p53 (compare Figs 2C and 4B). Complexes were reduced to 50% with the addition of 20 nM of HMG I(Y) at all concentrations of hMSH2–hMSH6 tested. Further, it was found that HMG I(Y) also caused a dissociation of hMSH2–hMSH6 binding to the 3-cytosine bulge which is a preferred target lesion for this repair protein (Fig. 4B). hMSH2–hMSH6/DNA complexes with both probes were quantified, normalized to complex formation in the absence of HMG I(Y) and plotted as a function of HMG I(Y) concentration (Fig. 4B). It was found that the rate of dissociation of the 3-cytosine bulge complexes was slightly slower than those with the Holliday junction probe. Thus, while the HMG I(Y) effect on p53 and hMSH2–hMSH6 binding is similar for the Holliday junction template, it is quite different with the 3-cytosine bulge substrate.

hMSH2–hMSH6 enhances p53 complex formation with Holliday junctions and 3-cytosine bulges

In these studies, it was found that HMG I(Y), a protein that binds four-way junction structures, competes with both p53 and hMSH2–hMSH6 for this substrate. These results imply that HMG I(Y) has a higher affinity for Holliday junctions than the other two proteins or places the DNA in a conformation unfavorable for p53 and hMSH2–hMSH6 binding. It is also possible that the latter proteins are more labile and readily associate and dissociate with DNA. To further explore the nature of p53 and hMSH2–hMSH6 complexes with four-way junctions and 3-cytosine bulges, the interaction between these proteins on the two probes was tested. In gel retardation assays the two proteins show different mobilities, so the respective complexes could be easily distinguished (Fig. 5A, compare lane 3 with lane 6). When both proteins were present, hMSH2–hMSH6 promoted p53 binding to the Holliday junction (compare lane 3 with 8, lane 4 with 9 and lane 5 with 10). These results indicate that hMSH2–hMSH6 enhances p53 binding to Holliday junctions

Figure 4. HMG I(Y) effects on hMSH2–hMSH6 complexes from Holliday junction and 3-cytosine bulge templates. (A) HMG I(Y) dissociates hMSH2–hMSH6 complexes from Holliday junctions and 3-cytosine bulges. Holliday junction, duplex or 3-cytosine bulge probes (7.5 nM) were incubated with hMSH2–hMSH6, HMG I(Y) or both proteins at room temperature for 20 min. The reaction products were separated by non-denaturing polyacrylamide gel electrophoresis followed by autoradiography. Protein concentrations are indicated above each lane. Arrows and brackets indicate the positions of the probe, hMSH2–hMSH6/DNA complexes and HMG I(Y)/DNA complexes. (B) HMG I(Y) causes dissociation of hMSH2–hMSH6 complexes from Holliday junction probes at the same rate as the 3-cytosine bulge probes. Probes containing 3-cytosine bulges or Holliday junctions (7.5 nM) were incubated with 75 nM hMSH2–hMSH6 in the presence of increasing amounts of HMG I(Y) followed by gel retardation assays. Protein/DNA complexes were quantified and plotted as a function of HMG I(Y) concentration.

and appears to assist in loading p53 on the template. On the other hand, in the presence of p53, there is a decrease in the hMSH2–hMSH6/DNA complexes (compare lane 6 with lane 10). Free probe was detected in reactions containing both proteins, indicating that the loss of hMSH2–hMSH6 complexes is due to dissociation by p53 and not lack of substrate DNA. The p53/DNA complexes in the absence and presence of increasing amounts of hMSH2–hMSH6 were quantified and the results revealed a 4–5-fold increase in p53 binding to the Holliday junction probe when hMSH2–hMSH6 was added (Fig. 5B). The interaction of the two proteins on the 3-cytosine bulge substrate was also tested (Fig. 5C). Similar to the Holliday junction substrate, there was an increase in the p53/DNA complexes with the addition of hMSH2–hMSH6, with a concomitant decrease in the hMSH2–hMSH6/DNA complexes. Thus, in contrast to HMG I(Y), p53 binding activity on both Holliday junctions and 3-cytosine bulges is enhanced by hMSH2–hMSH6.

Figure 5. hMSH2-hMSH6 effect on p53 binding to Holliday junction and 3-cytosine bulge templates. (A) hMSH2-hMSH6 induces p53 complex formation with Holliday junction substrates. Holliday junction probes (7.5 nM) were incubated with either p53 or hMSH2-hMSH6 or both proteins at concentrations indicated above each lane and complexes separated from free probe by gel retardation assays. (B) Quantification of p53 complexes with Holliday junctions in the presence of hMSH2-hMSH6. Holliday junction probes (7.5 nM) were incubated with 75 nM p53 and 0, 17, 34, 51 and 65 nM hMSH2-hMSH6 and complexes separated by gel retardation assays ($n = 4$). p53/DNA complexes were quantified and the fold increase in complex formation was plotted as a function of hMSH2-hMSH6 concentration. (C) hMSH2-hMSH6 induces p53 complex formation with 3-cytosine bulges. Holliday junction probes (7.5 nM) were incubated with either p53 or hMSH2-hMSH6 or both proteins at concentrations indicated above each lane and complexes separated from free probe by gel retardation assays.

DISCUSSION

Lesion binding by p53 represents an important biological feature of this protein and, to date, p53 has been shown to bind a range of damage intermediates including 3-cytosine bulges and Holliday junctions as well as single and double strand breaks. The role of damage recognition in downstream pathways is not clear. One possibility is the activation of p53 sequence-specific binding to enable transactivation of various gene products that play a key role in cell cycle arrest and apoptosis. Additionally, p53 interactions might facilitate direct recruitment of repair and recombination proteins for resolution of these lesions. Finally, binding to damage sites may ensure that p53 is in close proximity to protein kinases that can activate p53 by phosphorylating specific serine residues. Recent studies have argued against allosteric regulation of p53 sequence-specific binding *in vivo* (49); thus, damage binding may play a more important role in recruitment of repair factors or in events leading to post-translational modifications of p53.

The multiple roles of p53 in DNA recognition and binding necessitate tight regulation of this protein by a host of factors to ensure proper interactions at various recognition sites. In this study, the effect of three different proteins on p53 binding to Holliday junctions and extra base bulges was examined. Two members of the HMG group of proteins, HMG-1 and HMG I(Y), and the mismatch repair protein, hMSH2-hMSH6, were tested. The binding properties of p53 to these substrates are modulated in different ways by these proteins. We found that HMG-1 had no effect on p53 binding to either substrate. On the other hand, HMG I(Y) reduced p53 and hMSH2-hMSH6 binding to Holliday junction templates. Interestingly, HMG I(Y) also dissociated hMSH2-hMSH6 from 3-cytosine bulges but did not affect p53 binding to the same template. Finally, hMSH2-hMSH6 enhanced p53 complex formation with both templates.

The dissociation of p53 and hMSH2-hMSH6 from Holliday junction structures by HMG I(Y) may be a result of alteration of DNA conformation. Holliday junctions can adopt two different conformations—the stacked X structure and the square open form. In the presence of divalent cations, the four-way junctions adopt the stacked X conformation with 60° and 120° angles between the arms, whereas in the absence of divalent cations the junctions are in the square open form with 90° angles between the arms. HMG I(Y) has been shown to preferentially bind the open form of the four-way junction and maintain the junctions in this configuration (50). Previous studies from our laboratory have suggested that p53 may prefer to bind the DNA in the stacked X conformation as opposed to the open form (16). This was determined by electron microscopy where a large percent of the protein bound Holliday junctions adopted a conformation resembling the stacked X structure. HMG I(Y) binding could be causing a conformation change in the Holliday junction making it an unfavorable substrate for p53. Our studies also show that HMG I(Y) can dissociate p53/Holliday junction complexes that have been pre-formed. It is possible that p53 actively associates and dissociates from the junction crossover and, when dissociated, HMG I(Y) could rapidly bind the junction, altering its conformation, thus preventing p53 re-association. On the other hand, the dissociation of both p53 and hMSH2-hMSH6 complexes from Holliday junctions may be due to a direct influence of HMG I(Y) on both proteins.

HMG-1 has been shown to interact with p53 using far western assays by Jayaraman *et al.* (25). However, we were unable to detect similar interactions between p53 and HMG I(Y) using the same assay (data not shown). Additionally, immunoprecipitation assays using purified proteins did not reveal any direct interactions between p53 and HMG I(Y) even in the presence of DNA substrates (data not shown). Thus, the dissociation of p53 and hMSH2-hMSH6 from Holliday junction templates by HMG I(Y) appears to be a result of the differing binding affinities of these proteins.

The ability of HMG I(Y) to displace proteins from four-way junction substrates has been demonstrated previously. Four-way junction substrates were used to mimic linker DNA near the entrance and exit points of nucleosomes and histone H1 was shown to bind with high affinity to these structures. However, when HMG I(Y) was added there was an active dissociation of H1 from these templates (47). It was suggested that since HMG I(Y) plays an important role in genetic

recombination and retroviral integration, it dissociates H1 from these structures to allow recombination events to proceed. This may occur by direct recruitment of proteins for junction resolution or HMG I(Y) may act as a marker to signify the location of the four-way junction. Displacement of p53 and hMSH2–hMSH6 from Holliday junctions by HMG I(Y) may occur for similar reasons. In the event of DNA damage by environmental factors there is an increase in the formation of four-way junctions due to illegitimate sister chromatid exchange. In the cell, p53, hMSH2–hMSH6 and HMG I(Y) can bind to these four-way structures. We recognize that initial recognition of lesions would primarily be carried out by hMSH2–hMSH6 and p53 interactions with such sites of damage may provide a backup mechanism for signaling repair. The HMG proteins are more abundant in cells, hence, HMG I(Y) dissociation of p53 and hMSH2–hMSH6 from certain lesions or structures would be expected to dominate. However, following DNA damaging events the levels and stability of p53 and hMSH2–hMSH6 may alter, causing changes in the HMG I(Y) effect. Genetic studies altering HMG protein levels may be complicated to interpret with regard to, for example, p53-mediated genomic stability or hMSH2–hMSH6 catalyzed repair, due to the multiple pathways that are involved. These considerations underscore the need for further studies involving multiple proteins as an approach to learn how lesions are signaled in the cell.

While p53/DNA lesion complexes form with high affinity it is possible that the protein is quite labile on the DNA, thus HMG I(Y) is readily able to dissociate these interactions. The ability of p53 to form more stable complexes with DNA may require the protein to be post-translationally modified. Several studies have shown that p53 binding properties are modulated by post-translational modifications such as phosphorylation and acetylation. Purified p53 used in these studies was obtained from baculovirus-infected insect cells and was determined to be phosphorylated on at least one site. Further phosphorylation and/or acetylation may result in conformation changes in p53 that will enable more stable binding. Indeed, as mentioned above, p53 binding to lesions may function to bring the protein in close proximity to various kinases. Studies are currently underway in our laboratory to test the effect of phosphorylating various serine residues with specific kinases on the lesion binding properties of p53. It should be noted that in the presence of hMSH2–hMSH6, p53 complexes with lesions is enhanced, suggesting that protein interactions in the vicinity of the lesion dictate DNA binding rather than the lability of p53.

The effect of hMSH2–hMSH6 on p53/Holliday junction complexes we observed here was in direct contrast to that of HMG I(Y), where the repair protein had a stimulatory effect on p53 binding. The increase in p53 complexes also results in a concomitant decrease of the hMSH2–hMSH6 complexes with the same substrate. This reduction was seen even when molar excesses of hMSH2–hMSH6 were added to the reactions (data not shown). Additionally, when hMSH2–hMSH6 was pre-incubated with the substrates followed by the addition of p53, enhancement of the p53/DNA complexes was observed (data not shown). Similar effects were seen with the 3-cytosine bulge template that is a preferred substrate for hMSH2–hMSH6. Hence it appears that hMSH2–hMSH6 is also able to load p53 on the 3-cytosine bulge substrate. It is possible that p53 and hMSH2–hMSH6 interact

with each other at the lesion sites. However, if this were the case, we would expect a novel band containing a ternary complex with both proteins to appear on the gel retardation assays. No such complexes were detected in the presence of both proteins with either probe used. Furthermore, immunoprecipitation assays did not reveal any interaction between the two proteins in solution (data not shown). Thus, hMSH2–hMSH6 appears to be either recruiting p53 to lesion sites or stabilizing p53/DNA interactions in some manner. This may occur to ensure that the lesion is signaled to the necessary downstream proteins and cell cycle arrest or apoptosis pathways are executed.

In the nucleus, DNA exists in several different configurations and a range of proteins can bind these various forms. In this paper, we examined the dynamics between three proteins that bind Holliday junctions and have shown that they interact with each other in different ways. p53 binding to these recombination intermediates is affected in opposite ways by HMG I(Y) and hMSH2–hMSH6—HMG I(Y) dissociates while hMSH2–hMSH6 promotes binding to Holliday junctions and 3-cytosine bulges. Presumably, in the cellular environment, other proteins will further contribute and dictate specific interactions of these proteins on the DNA.

ACKNOWLEDGEMENTS

This work was supported in part by National Institutes of Health Grants GM31819 and CA70343. We thank members of the Griffith laboratory for helpful discussion, and Dr Arnold Levine, Dr Richard Fishel and Dr Michael Bustin for providing vectors for protein purification.

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