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High Affinity MAR-DNA Binding Is a Common Property of Murine and Human Mutant p53

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Abstract We recently reported that murine MethA mutant but not wild-type p53 specifically binds to MAR-DNA elements (MARs) with high affinity. Here we show that this DNA binding activity is exerted not only by MethA mutant p53 but also by other murine mutant p53 proteins isolated from the transformed murine BALB/c cell lines 3T3tx and T3T3 and differing in their conformational status. High affinity MAR-DNA binding was not restricted to the *XbaI*-IgE-MAR-DNA fragment from the murine immunoglobulin heavy chain gene enhancer locus [Cockerill et al. (1987): J Biol Chem 262:5394–5397] used in previous studies, as MethA p53 also specifically interacted with other A/T-rich bona fide MARs. Not only murine but also human mutant p53 proteins carrying the mutational hot spot amino acid exchanges 175Arg→His, 273Arg→Pro, or 273Arg→His bound to the *XbaI*-IgE-MAR-DNA fragment. We therefore conclude that high affinity MAR-DNA binding is a property common to a variety of mutant p53 proteins. J. Cell. Biochem. 69:260–270, 1998.

Key words: oncogenic function of mutant p53; MAR-DNA elements; MAR-DNA binding by mutant p53; MethA p53

Point mutations and more rarely rearrangements in the tumor suppressor gene p53 so far are the most common genetic alterations in human cancer [Vogelstein and Kinzler 1992; Hollstein et al., 1996; Hainaut et al., 1997]. These mutations inactivate the tumor suppressor functions of p53, most significantly its function as a transactivator of p53 target genes after genotoxic stress, due to loss of its ability to interact with p53 consensus DNA elements in a sequence-specific manner. However, in contrast to other classic tumor suppressors, mutant p53 proteins have not simply lost the wild-type specific tumor suppressor functions but display an oncogenic potential of their own [Deppert, 1994, 1996]. The molecular basis for this gain of function of mutant p53 is still unclear, but it seems to require an intact transactivation domain of the p53 molecule [Deppert, 1996; Lin et al., 1995]. In line with this observation, it has been shown that mutant p53 can upregulate the expression of several genes with functions in tumor progression, such as the mdr-1, the PCNA, or the VEGF genes [Deppert, 1996; Cox and Lane, 1995], but this postulated mutant p53–specific transactivator function still is controversially discussed [Deppert, 1996]. In any case, mutant p53–specific transactivation must occur by a different mechanism than the transactivation of p53 target genes by wild-type p53, as no consensus sequence specifically recognized by mutant p53 could be identified.

In search of a specific interaction of mutant p53 with DNA which differs from that of wild-type p53, we previously reported that the oncogenic double mutant p53 protein (amino acids 168Glu→Gly, 234Met→Ile) purified from MethA cells, a methylcholanthrene-induced mouse tumor cell line [DeLeo et al., 1977], bound to the *AluI*-λ-DNA fragment [Weiβker et al., 1992]. Not only did this fragment exhibit properties of matrix attachment region DNA elements (MARs), but the binding characteristics of mutant p53 to this DNA fragment were similar to that of other bona fide MAR-DNA binding pro-

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teins [Weiβker et al., 1992]. Consequently it was found that MethA p53 also bound to MAR-DNA fragments from the murine immunoglobulin heavy chain gene enhancer locus (*XbaI*-IgE-MAR-DNA fragment) [Cockerill et al., 1987] and the interferon β gene locus in a specific manner [Weiβker et al., 1992; Müller et al., 1996]. The MAR-DNA binding activity of mutant p53 is clearly distinguishable from other DNA binding activities such as the sequence-specific DNA binding of wild-type p53 or the nonspecific binding to double- and single-stranded DNA and RNA, exhibited both by wild-type and mutant p53 [Steinmeyer and Deppert, 1988; Kern et al., 1991; Bakalkin et al., 1995]. As MARs exert important regulatory functions in gene expression [Bode and Maaβ, 1988], MAR-DNA binding by mutant p53 could form the molecular basis for mutant p53 specific transactivation and thereby possibly for the gain of function phenotype of at least some mutant p53 proteins.

An important question regarding the relevance of the MAR-DNA binding activity observed with murine MethA mutant p53 protein was whether this interaction would be exhibited also by other mutant p53 proteins. Furthermore, it was important to analyze whether mutant p53 would interact with a variety of different MARs. Here we present data demonstrating that not only MethA p53 but also other murine mutant p53 proteins (from 3T3tx and T3T3 cells [Milner et al., 1993]) as well as the human hot spot mutant p53 proteins (175Arg→His, 273Arg→His, 273Arg→Pro) bind to several A/T-rich bona fide MARs with much higher affinity than wild-type p53. High affinity MAR-DNA binding therefore seems to be a common property of mutant p53.

MATERIALS AND METHODS

Isolation of p53 Proteins

Baculoviral expression and preparation of p53 protein from insect cells (method A). High five™ insect cells at 80% confluence were infected with recombinant baculovirus expressing wild-type (murine, human) or mutant human 273Arg→Pro or murine MethA p53. Forty-eight hours after infection, the cells were harvested, washed four times with phosphate-buffered saline (PBS) at 4°C, resuspended in extraction buffer A (10 mM HEPES, pH 7.4; 1.5 mM $MgCl_2$; 5 mM KCl) and incubated on ice for 60 min. To avoid protein degradation, we carried out all following steps on ice, and 10 mM DTT and protease inhibitors (2 mM PMSF, 1% (v/v) trasylol, 0.05% (w/v) leupeptin) were added to the extraction buffers. The cells were lysed using a dounce-homogenizer (30 strokes), incubated for 45 min, and collected by centrifugation at 5,000*g* at 4°C for 10 min. The supernatant A was removed and collected, and the cell pellet was resuspended in buffer B (10 mM HEPES, pH 9.0; 1.5 mM $MgCl_2$; 5 mM KCl), incubated 45 min, and centrifuged at 5,000*g* at 4°C for 10 min, yielding supernatant B. The pellet was extracted twice using buffer B containing 0.2 or 0.5 M KCl, respectively, resulting in supernatants C and D. Supernatants A, B, C, and D were cleared by centrifugation at 10,000*g* for 30 min at 4°C and stored at -70°C. The protein pattern of these fractions was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the p53 content was determined by Western blotting using a polyclonal sheep anti-p53 serum (Boehringer Mannheim, Germany) and affinity-purified, peroxidase-conjugated antisheep goat IgG (Sigma, St. Louis, MO). p53 bands were visualized by chemoluminescence enhancement using the Super-Signal™ ULTRA Chemoluminescent system (Pierce, Rockford, IL). p53 protein from fraction C was approximately 80% pure and was subsequently subjected to the MAR-DNA binding experiments.

Extraction of p53 protein from murine cells and bacteria expressing recombinant p53 protein (method B). BALB/c T3T3 cells [Yewdell et al., 1986; Milner and Medcalf, 1991] and BALB/c 3T3tx [Milner and Medcalf 1991; Milner and Cook, 1986] were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). MethA cells [DeLeo et al., 1977] were grown in DMEM containing 5% FCS. Cells were harvested, washed twice with 1× PBS, and extracted for 30 min on ice in lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% NP40, 10% (v/v) glycerol). Extracts were cleared by centrifugation at 12,000*g* for 15 min at 4°C. Lysates from bacteria expressing the respective p53 protein (human mutant p53 273Arg→His) were prepared following standard procedures [Mummenbrauer et al., 1996]. Human wild-type p53 cDNA and human 273Arg→His p53 cDNA (mutant cDNA was provided by H.-W. Stürzbecher,

Institut für Humangenetik, Lübeck, Germany) were inserted into the pET-19b plasmid and expressed in BL21 (DE3) bacteria [Studier et al., 1990]. About 50% of the overexpressed wild-type p53 and 20% of the overexpressed mutant p53 were soluble. After overexpressing p53, bacteria were lysed by addition of ice-cold lysis buffer (20 mM Tris, pH 8.0, 2 mM EGTA, pH 8.0, 0.5% Lubrol, 500 mM NaCl, 10 mM DTT, 2 mM PMSF, 5 mM $Na_2S_2O_5$). Crude lysates were centrifuged at 200,000*g* for 1 h to remove cellular debris. The clarified extracts containing the respective p53 proteins were subjected to PAb248 antibody affinity columns. Antibody-p53 complexes linked to protein A-Sepharose (PAS) were washed with buffer A (30 mM KPi, pH 8.0, 50 mM KCl, 1 mM EDTA, 2 mM DTT) and eluted with buffer A containing 1 M KCl and subsequently with buffer B (100 mM KPi, pH 12, 1M KCl, 1 mM EDTA, 2 mM DTT), followed by immediate neutralization with KH_2PO_4. Aliquots of the eluates were subjected to SDS-PAGE, and their concentrations were estimated after Coomassie blue staining in comparison to marker proteins of known concentration. Western blotting was carried out as described above.

Characterization of p53 Proteins by Immunoprecipitation Using Conformation-Specific Antibodies

Aliquots of the isolated proteins were diluted with lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% NP40, 10% (v/v) glycerol, 5 mM DTT) and mixed with 30 µl of settled protein A-Sepharose (Pharmacia, Freiburg, Germany). Immunoprecipitation and analysis of the immunoprecipitated p53 by SDS-PAGE were carried out as described previously [Staufenbiel and Deppert, 1983]. Wild-type p53 was immunoprecipitated with monoclonal antibody PAb421 as well as with antibody PAb1620, the latter recognizing p53 in the wild-type conformation only. Immunoprecipitation of mutant p53 was performed using monoclonal antibody PAb421 and mutant conformation-specific antibody PAb240. Proteins were separated on an 11% SDS-polyacrylamide gel, transferred to Hybond-C super membranes (Amersham, Braunschweig, Germany), immunodetected by polyclonal anti-p53 serum from sheep (Boehringer Mannheim), and visualized as described above.

Isolation of the K28- and K19C- MAR-DNA Fragments

These MAR-DNA sequences were identified by cloning of the MAR-DNA (1.7% total) after digestion and removal of chromatin loop DNA with micrococcal nuclease as described [Boulikas and Kong, 1993]. The K19C-MAR-DNA fragment is a subfragment of the 3.6 kb MAR from the human choline acetyltransferase gene locus that also displays MAR activities and acts as origin of replication.

Liquid Phase Binding Assay

The respective MAR-DNA fragments (the *XbaI*-IgE-MAR-DNA fragment cloned into pUC19 vector, the *BamHI/HindIII*-K28-MAR-DNA fragment and the *PstI/EcoRI*-K19C-MAR-DNA fragment cloned into Bluescript SK (+/-) phagemid) and the A/T-rich *EcoRI/BamHI*-non-MAR-DNA fragment from the chicken lysozyme locus [von Kries et al., 1991] cloned into the multiple cloning site of pUC19 vector were isolated by restriction enzyme digest from the vector DNA and separated by gel electrophoresis, purified from the gel, and end-labeled using T4 polynucleotide kinase and (γ-32-P) ATP according to standard procedures. For MAR-DNA binding, equal amounts from each p53 preparation (approximately 200 ng) were transferred into the binding buffer (SWB buffer: 50 mM NaCl, 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 6 mM $MgCl_2$, 0.02% BSA, 0.02% polyvinylpyrolidone [Müller et al., 1996]), including the desired radioactively labeled MAR-DNA fragments and unlabeled nonspecific competitor DNA (1,118 bp *BglI* puC19 fragment), and incubated for 30 min at room temperature. During the competition experiments with specific competitor DNA, varying amounts of the 997 bp *XbaI*-IgE-MAR-DNA fragment were added. Subsequently, antibody PAb248 (murine) or PAb1801 (human) and PAS were added and the assay mixture kept in an Eppendorf shaker for 30 min at room temperature. The DNA-protein-antibody-PAS complexes then were washed three times with SWB buffer, and bound DNA fragments were quantitatively eluted with 500 µl of 100 mM ammonium hydrogen carbonate, pH 9.5, for 45 min at 37°C. The eluates were lyophilized and dissolved in 20 µl of gel loading buffer (water, 10% glycerol, bromophenol blue). Samples were subjected to DNA-SDS-PAGE and visualized by autoradiography.

RESULTS

Human Mutant p53 Does Not React With MAR-DNA Fragments in a South-Western DNA Binding Assay

We first compared the MAR-DNA binding activities of different mutant p53 proteins, three murine (MethA, 3T3tx, T3T3) (Table I) and three human mutant p53 proteins (175Arg→His, 273Arg→Pro, 273Arg→His) derived from different cellular and recombinant sources (see Table I and Materials and Methods) with that of the respective wild-type p53 proteins in the South-western DNA binding assay described previously [Müller et al., 1996]. Murine mutant and wild-type p53 proteins were isolated from the respective cell lines (BALB/c mouse T3T3, 3T3tx, and MethA cells), or from baculovirus-infected insect cells (murine wild-type p53, MethA p53). Human p53 proteins were purified from bacteria expressing recombinant p53 (273Arg→His) and from baculovirus-infected insect cells (wild-type p53, mutant p53 proteins 273Arg→Pro and 175Arg→His) (see Materials and Methods). The purity and conformational status of the isolated p53 proteins was checked by immunoprecipitation and Western blotting (see Materials and Methods), and aliquots of the p53 proteins were applied to MAR-DNA binding analysis using the radioactively labeled *XbaI*-IgE-MAR-DNA fragment, following the protocol for the recently described South-western binding assay [Müller et al., 1996]. In accordance with our previous findings [Müller et al, 1996], murine mutant p53 proteins in repeated experiments bound the IgE-MAR-DNA fragment with a much higher affinity than wild-type p53 in the presence of a high molar excess (1,000-fold) of nonspecific competitor DNA. In contrast, quite varying results were obtained with the human mutant p53 proteins, ranging from weak to no binding at all (data not shown). Rather than assuming that MAR-DNA binding is a property specific for murine mutant p53, we considered the possibility that the apparent lack of a reproducible MAR-DNA binding activity of human mutant p53 in the South-western assay reflected structural differences between human and murine p53. Although human and murine p53 share extensive homologies, these proteins exhibit sequence and conformational differences [Soussi and May, 1996]. The most critical step in the South-western binding assay is the renaturation of the blotted p53 protein, reestablishing the ability of p53 to specifically interact with DNA. Therefore, we suspected that problems in refolding the human p53 proteins accounted for our difficulties to reproducibly demonstrate MAR-DNA binding for human mutant p53 proteins in this assay. Due to these intrinsic limitations of the South-western binding assay for the analysis of MAR-DNA binding by human p53, we developed an assay which did not require a renaturation step and allowed the analysis of MAR-DNA binding of native murine and human wild-type and mutant p53 proteins.

Murine MethA Mutant p53 Specifically Binds to A/T-Rich MAR-DNA Elements in a Liquid-Phase Binding Assay

As a suitable binding assay, we adapted the McKay [1981] liquid-phase binding assay for

TABLE I. Characteristics of the p53 Proteins Analyzed for MAR-DNA Binding*

p53 protein	Genotype	Amino acid change	Reactivity with PAb240	Reactivity with PAb1620/246
Wild-type	Normal	none	−	+
T3T3	230	tyr to asp	−	+
	287	glu to asp		
	270	arg to cys		
MethA	168	glu to gly	+	−
	234	met to ile		
3T3tx	230	tyr to asp	+	−
	237	met to ile		
175His	175	arg to his	+	−
273Pro	273	arg to pro	+	−
273His	273	arg to his	−	+

*Wild-type p53 proteins react with PAb246 (murine) and PAb1620 (human) but not with PAb240, whereas mutant p53 proteins are recognized by PAb240 but not by PAb246 and PAb1620. The listed genotypes of the T3T3 mutant p53 are based on the alleles published by Milner et al. [1993] (230 and 287) or Kolzau and Deppert [1993] (270).

the analysis of MAR-DNA binding by p53. This assay consists of three steps (for details see Materials and Methods): 1) incubation of the p53 protein with the ^{32}P–end-labeled MAR-DNA probe and the unlabeled competitor DNA to allow specific binding of the MAR-DNA, 2) formation of DNA/p53/antibody complexes using the p53-specific monoclonal antibodies PAb248 for murine or PAb1801 for human p53 (these antibodies recognize epitopes on the p53 N-terminus and thus do not interfere with MAR-DNA binding of mutant p53 [Weiβker et al., 1992; Müller et al., 1996]), and 3) separation and purification of these complexes after their immobilization with protein A-Sepharose (PAS). The bound MAR-DNA then was quantitatively eluted and analyzed by gel electrophoresis.

We applied this assay for the analysis of MAR-DNA binding of recombinant murine wild-type and MethA mutant p53 proteins to different MARs, first using the IgE-MAR. Figure 1A shows that in the absence of competitor DNA the IgE-MAR probe was bound by both p53 proteins but not by the antibody-PAS complexes alone (Fig. 1A, lane 1). In accordance with our previous findings, the specific high affinity binding of the IgE-MAR by MethA mutant p53 could be demonstrated in the presence of a high molar excess of nonspecific DNA (Fig. 1A, lane 6). Under these conditions, murine wild-type p53 failed to bind the IgE-MAR probe (Fig. 1A, lane 7). Similar results were obtained using the 393 bp K28-MAR-DNA fragment from the human PARP gene locus [Boulikas et al., 1996] (Fig. 1B, lane 6). While binding of wild-type p53 to the K28-MAR-DNA fragment was strongly reduced in the presence of a 1,000-fold molar excess of nonspecific competitor DNA (*BglI*-puC19-DNA fragment) (Fig. 1B, lane 7), equal amounts of nonspecific competitor DNA did not influence the binding of MethA mutant p53 to the K28-MAR-DNA fragment (Fig. 1B, lane 6). In contrast, the K19C-MAR-DNA fragment from the human choline acetyltransferase gene was bound with a much lower affinity by MethA mutant p53. Whereas at a 100-fold molar excess of nonspecific competitor DNA MethA p53 bound this fragment somewhat more specific than wild-type p53, binding of this MAR-DNA fragment by both wild-type and mutant p53 was effectively competed out by a 1,000-fold molar excess of nonspecific competitor DNA (Fig. 1C, lanes 6,7). The K19C-MAR-DNA fragment has an A/T content of 47%, which is considerably less than the A/T content of the strongly bound MAR-DNA fragments like the IgE-MAR-DNA fragment, the K28-MAR-DNA fragment, and the β interferon-MAR-DNA fragment, which have an A/T content of ≥60%. However, as previously demonstrated [Weiβker et al., 1992; Müller et al., 1996] , A/T richness as such is not sufficient to mediate binding to mutant p53, as neither wild-type nor MethA mutant p53 bound to an A/T-rich (59.5%) non–MAR-DNA fragment from the chicken lysozyme locus [von Kries et al., 1991] also in this assay (Fig. 1D, lanes 2–9).

Analysis of MAR-DNA Binding Activities of Different Murine Mutant p53 Proteins

We next compared in the liquid-phase binding assay the MAR-DNA binding activity of murine mutant p53 proteins which differ in their conformational status on the basis of their immunoreactivity (wild-type: T3T3; mutant: MethA, 3T3tx) using the *XbaI*-IgE-MAR-DNA fragment as a probe. Figure 2 shows the results of binding reactions using these mutant p53 proteins and wild-type p53. MAR-DNA binding of wild-type p53 again was strongly reduced in the presence of a 100-fold excess of nonspecific competitor DNA (1,118 bp *BglI*-puC19-DNA fragment) over the IgE-MAR-DNA (Fig. 2A, lane 10). In contrast, the same amount of nonspecific competitor DNA only slightly influenced MAR-DNA binding by the MethA and T3T3 mutant p53 proteins (Fig. 2A, lanes 8,9). However, addition of a 1,000-fold molar excess of unlabeled nonspecific competitor DNA abolished MAR-DNA binding of mutant T3T3 p53 (Fig. 2A, lane 12), whereas MethA p53 still strongly bound the IgE-MAR (Fig. 2A, lane 11). Complete inhibition of MAR-DNA binding of MethA p53 required a much higher excess of unlabeled nonspecific competitor DNA (>10,000-fold) (data not shown). To rule out that these differences in MAR-DNA binding by different mutant p53 proteins reflected different nonspecific DNA binding activities of these p53 proteins, we performed competition experiments with specific competitor DNA (997 bp *XbaI*-IgE-MAR-DNA fragment), now also including the conformational mutant 3T3tx p53. Figure 2B shows that MAR-DNA binding by wild-type p53 was already reduced in the presence of a twentyfold molar excess of specific competitor DNA (Fig. 2B, lane 13), and completely abolished by the addition of a thirtyfold molar excess of cold IgE-MAR-DNA fragment (Fig. 2B, lane 18). Interestingly, a thirtyfold

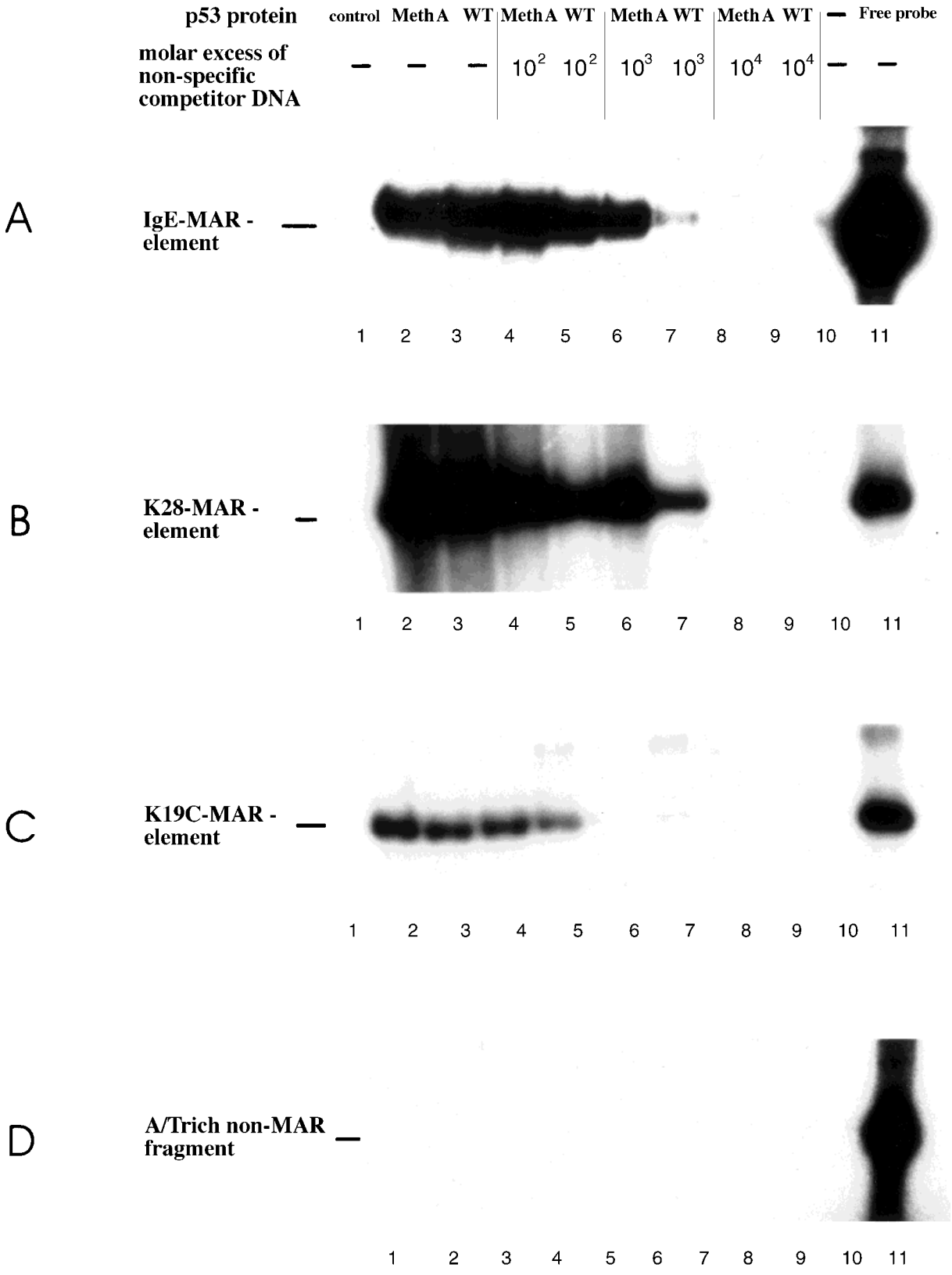


Fig. 1. Specific binding of murine MethA p53 protein to different MAR-DNA fragments. Equal amounts of recombinant murine MethA mutant p53 and wild-type p53 were subjected to the liquid-phase DNA binding assay as described in Materials and Methods, using different ^{32}P–end-labeled MAR-DNA fragments in the absence or presence of nonspecific competitor DNA. **A:** Analysis of the *XbaI*-IgE-MAR-DNA fragment (997 bp), **B:** The K28-MAR-DNA fragment from the human PARP gene locus (393 bp), **C:** The K19C-MAR-DNA from the human choline acetyltransferase gene locus (651 bp). **D:** An A/T-rich non–MAR-DNA fragment from the chicken lysozyme gene locus (600 bp) was used as a negative control. The MAR-DNAs bound by p53 were analyzed on 11% SDS polyacrylamide gels and visualized by autoradiography. The labeled probes were not bound by the antibody/protein A-Sepharose complexes (**lanes 1**). Binding of MethA and wild-type p53 proteins to the respective MAR-DNA fragments was analyzed in the absence (**lanes 2,3**) or in the presence of an increasing molar excess of unlabeled competitor DNA over ^{32}P-labeled MAR-DNA (**lanes 4–9**).

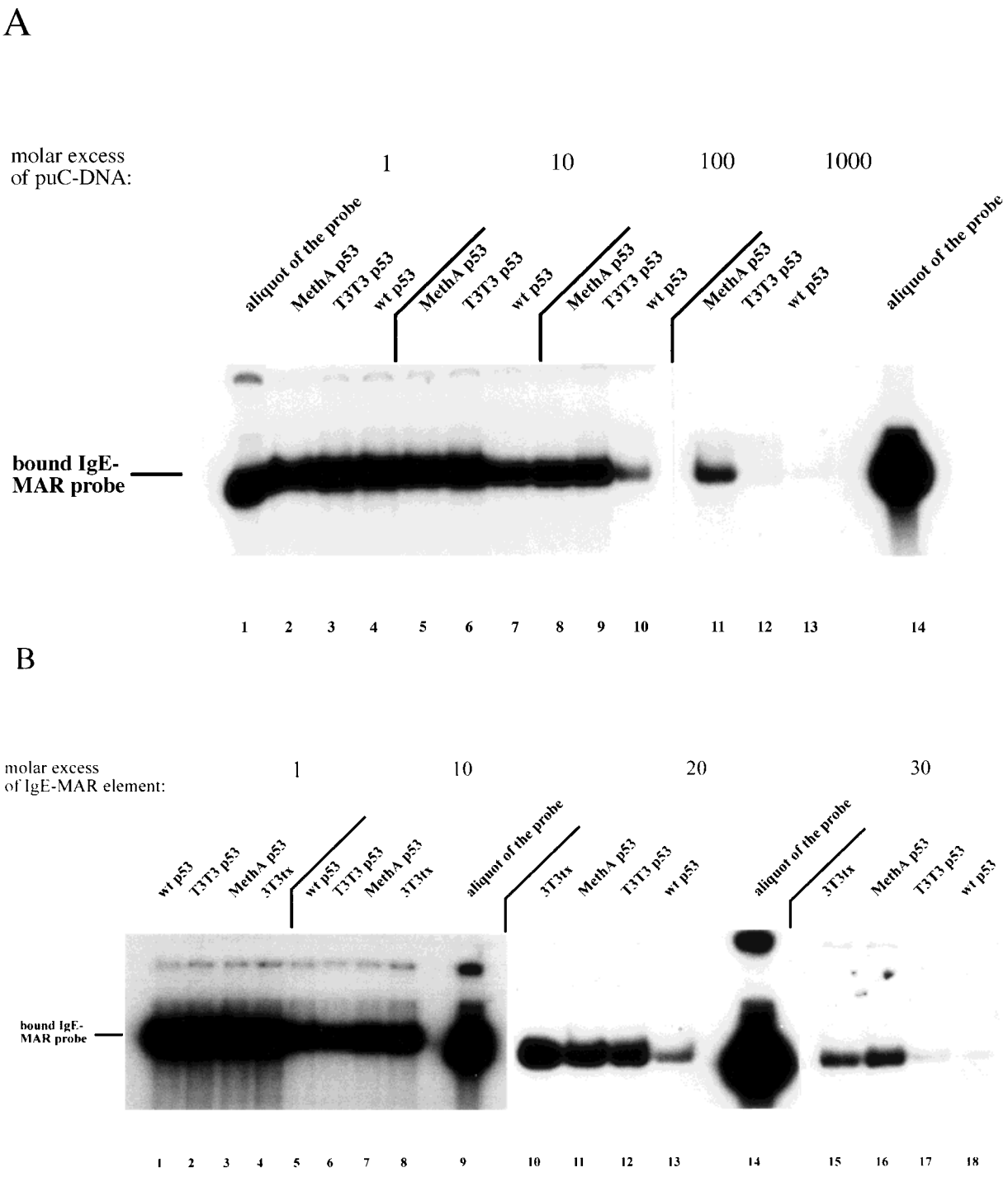


Fig. 2. Specific binding of different murine p53 proteins to the *XbaI*-IgE-MAR-DNA fragment. Equal amounts of different murine mutant p53 proteins (MethA, 3T3tx, T3T3) and wild-type p53 protein were analyzed for their binding to ^{32}P–end-labeled IgE-MAR-DNA in the presence of an increasing molar excess of (**A**) nonspecific competitor DNA (*BglI*-puC19 fragment) and (**B**) specific competitor DNA (IgE-MAR-DNA fragment) over ^{32}P-labeled IgE-MAR-DNA using the liquid-phase binding assay as described in Figure 1.

molar excess of cold specific competitor DNA also strongly reduced MAR-DNA binding of T3T3 mutant p53 (Fig. 2B, lane 17), in contrast to MAR-DNA binding of MethA p53 and of 3T3tx p53, which was still quite prominent under these conditions (Fig. 2B, lanes 15,16). The conformational wild-type T3T3 mutant p53 thus specifically binds to MARs but with a much lower affinity than the conformational mutant MethA and 3T3tx mutant p53 proteins.

Specific Binding of Human Mutant p53 Proteins to MAR-DNA Fragments

We next examined MAR-DNA binding of three different human mutant p53 proteins and of human wild-type p53 in the liquid-phase binding assay, again using the *XbaI*-IgE-MAR-DNA fragment as a probe. Mutant p53 proteins 175Arg→His and 273Arg→Pro, which are in mutant conformation [Ory et al., 1994], bound the IgE-MAR-DNA fragment even in the presence of a 1,000-fold molar excess of nonspecific competitor DNA (*BglI*-puC19-DNA fragment) (Fig. 3A, lane 6; Fig. 3B, lane 6), whereas binding of wild-type p53 was strongly reduced under the same conditions (Fig. 3A, lane 5; Fig. 3B, lane 7). Interestingly, also mutant p53 273Arg→His, described to retain wild-type conformation [Ory et al., 1994], bound the IgE-MAR-DNA fragment in the presence of a 1,000-fold molar excess of nonspecific competitor DNA (Fig. 3C, lane 7), which seems to be in apparent contrast to our data obtained with the conformational wild-type murine T3T3 mutant p53.

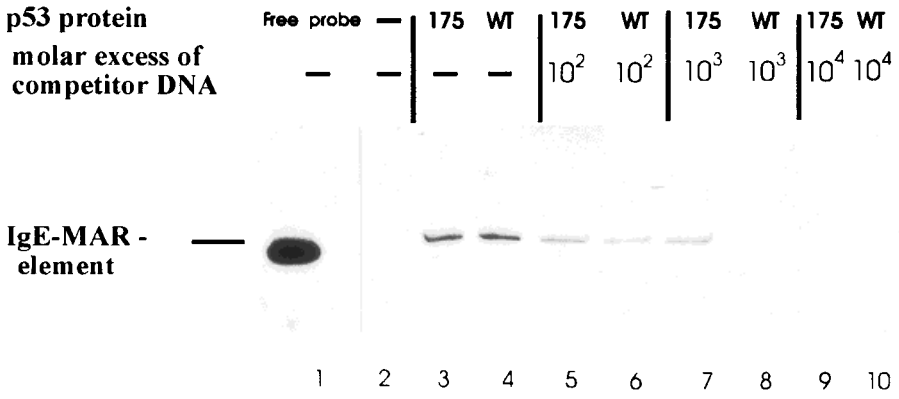
DISCUSSION

We here demonstrate that high affinity binding to MAR-DNA elements is a property common to murine and human mutant p53. This conclusion is supported by our finding that not only MethA p53 but also various murine (3T3tx, T3T3) and human mutant p53 proteins (175Arg→His, 273Arg→Pro, 273Arg→His) bound the IgE-MAR with a higher affinity than wild-type p53. Competition experiments using specific and nonspecific competitor DNA verified that MAR-DNA binding primarily is a mutant p53–specific activity, which is not mediated by the non-sequence-specific DNA binding activity exerted by both wild-type and mutant p53. Furthermore, the use of native proteins in the liquid-phase assay excluded the possibility that high affinity binding of mutant but not of wild-type p53 was due to the wild-type p53 protein being nonfunctional. However, it should be noted that wild-type p53 is not completely negative in MAR-DNA binding, as the binding of MARs by wild-type p53 was clearly of higher affinity than non-sequence-specific DNA binding. Whether the low affinity binding of wild-type p53 to MARs is of biological relevance or whether wild-type p53 binding to MAR-DNA elements is much more selective (i.e., whether it will also bind with high affinity to certain, so far not identified MARs) remains to be determined.

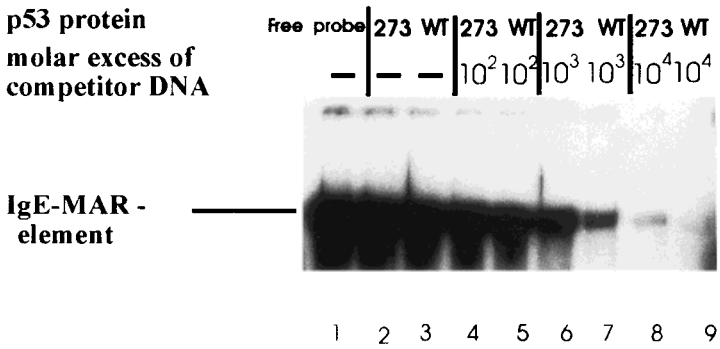
Despite our finding that high affinity MAR-DNA binding is a general property of mutant p53, some interesting differences were detected regarding the specificity of this activity both for a particular mutant p53 and for the MARs analyzed. First, not all MARs tested were bound with the same affinity by mutant p53. Whereas the IgE-MAR-DNA fragment and the K28-MAR-DNA fragment were bound with similar affinities by MethA mutant p53, binding to the K19C-MAR-DNA fragment was of much lower affinity. The latter MAR-DNA fragment differs from the others by a significantly lower A/T content (47% vs. >60%). However, A/T richness as such is not the sole determinant for MAR-DNA binding of mutant p53, as neither wild-type nor mutant p53 reacted with an A/T-rich non–MAR-DNA fragment. Therefore, it will be of interest to find out which determinant(s) on MAR-DNA is required for high affinity MAR-DNA binding by mutant p53.

The other interesting observation is our finding that the conformational wild-type T3T3 mutant p53 bound to the IgE-MARs with a considerably lower affinity than the other murine mutant p53 proteins tested, the conformationally mutant MethA and 3T3tx mutant p53 proteins. Again, competition experiments confirmed that the lower affinity of T3T3 mutant p53 for MAR-DNA did not reflect reduced nonspecific DNA binding of this mutant p53 as compared to the others. The comparison of the MAR-DNA binding activities of different human mutant p53 proteins then suggested that the lower MAR-DNA binding activity of T3T3 mutant is not due to this mutant p53 displaying a wild-type conformation, as the 273Arg→His mutant p53 bound the IgE-MAR-DNA fragment with similar affinity as the 175Arg→His and the 273Arg→Pro mutants. Whereas the 175Arg→His and the 273Arg→Pro mutants are functionally and conformationally mutant proteins, the 273Arg→His mutant is in a wild-type conformation and even has retained some functional properties of wild-type p53, especially after heterooligomerization with wild-type p53 [Ory et al., 1994]. Thus, having retained a wild-type conformation does not seem to account for the reduced MAR-DNA binding of the T3T3 mutant p53. An important difference between the 273Arg→His mutant and the T3T3 mutant p53, however, is that the latter one is defective in oligomerization [Milner et al., 1993], possi-

A 175 Arg→His



B 273 Arg→Pro



C 273 Arg→His

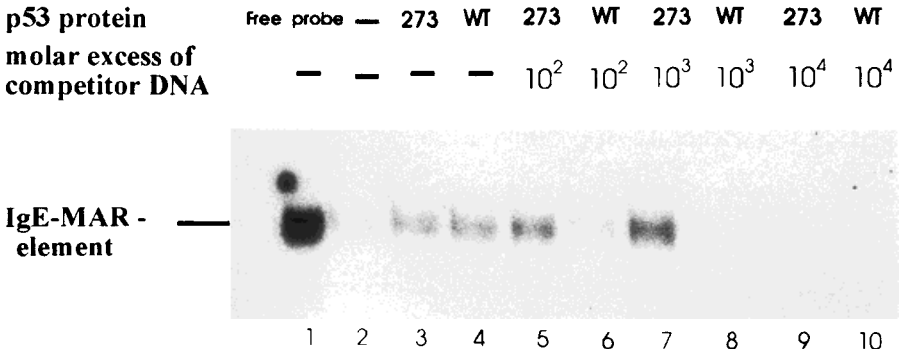


Fig. 3 Specific binding of different human p53 proteins to the *XbaI*-IgE-MAR fragment. Equal amounts of the human 175Arg→His, 273Arg→His, 273Arg→Pro mutant p53 proteins and wild-type p53 were analyzed for IgE-MAR-DNA binding in the presence of an increasing molar excess of nonspecific competitor DNA (*BglI*-puC19-DNA fragment) over ^{32}P-labeled IgE-MAR-DNA fragment using the liquid-phase binding assay as described in Figure 1. **A:** 175Arg→His mutant p53, **B:** 273Arg→Pro mutant p53, **C:** 273Arg→His mutant p53.

bly indicating that oligomerization is required for mutant p53 MAR-DNA binding. Another less likely explanation for the different behavior of these mutants is that T3T3 mutant p53, like 3T3tx mutant p53, had been isolated from mammalian cells, whereas the 273Arg→His mutant p53 was purified from recombinant bacteria. However, in all our experiments so far, we had been unable to detect differences in MAR-DNA binding of murine MethA mutant p53 purified from different sources (recombinant from bacteria and from baculovirus infected insect cells, and from MethA cells) [Müller et al., 1996]. Similarly, wild-type p53 purified either from insect cells infected with the respective recombinant baculovirus or from recombinant bacteria did not differ in their low affinity interactions with MARs (data not shown).

Mutations in codons 175 (Arg), 273 (Arg), and 248 (Arg) account for 22% of all p53 mutations found in human cancer [Ory et al., 1994]. Given the possibility of a functional relationship between high affinity MAR-DNA binding and the postulated oncogenic potential of mutant p53, it may be quite important that also the human 175Arg→His, 273Arg→Pro, or 273Arg→His p53 mutants exhibited high affinity MAR-DNA binding. However, the biological relevance of the MAR-DNA binding activity is not yet proven, and many questions remain to be resolved before MAR-DNA binding of mutant p53 can be related to its oncogenic activities. To further understand the molecular consequences of such interactions within tumor cells, we in a next step must identify the structural features within MAR-DNA which mediate the specific interaction of mutant p53 with these DNA elements. Despite these uncertainties, the exciting possibility emerges that high affinity MAR-DNA binding of mutant p53 may form the molecular basis for its oncogenic potential. MARs are located close to or include important regulatory elements such as enhancers and origins of replication [reviewed by Boulikas, 1995a,b]. MARs themselves can enhance up to 1,000-fold the expression of foreign genes in transgenic animals and plants as well as in stably transfected cells in culture insulating the transgene from position effects exerted from neighboring chromatin at the integration site [reviewed by Boulikas, 1995a,b]. Further understanding of the interaction of mutant p53 with MAR-DNA elements thus might open the possibility to abrogate the oncogenic functions of mutant p53 in tumor cells by interfering with this activity.

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