DNA strand breaks are potential interaction sites for the nuclear enzyme poly(ADP-ribose) polymerase (PARP; E.C. 2.4.2.30) and the tumor suppressor protein p53. Both proteins bind and respond to DNA breaks and both play a role in DNA damage signaling. A temporary colocalization and complex formation between these proteins has been demonstrated in mammalian cells. Here we show that free and poly(ADP-ribose) polymerase-bound ADP-ribose polymers target three domains in p53 protein for strong noncovalent interactions. The polymer binding sites could be mapped to two amino acid sequences in the sequence-specific core DNA binding domain of p53 (amino acid positions 153–178 and 231–253) and another one in the oligomerization domain (amino acids 326–348). In mobility shift experiments, poly(ADP-ribose) effectively prevented and reversed p53 binding to the palindromic p53 consensus sequence. Additionally, poly(ADP-ribose) also interfered with the DNA single strand end binding of p53. The results suggest that ADP-ribose polymers could play a role in regulating the DNA binding properties of p53.

The nuclear enzyme PARP1 and the tumor suppressor protein p53 are both involved in DNA damage signaling. Both bind to and are activated by DNA strand breaks, albeit in a different manner. The binding of PARP involves two zinc fingers in the N-terminal DNA binding domain, and this activates the four catalytic activities in the C-terminal domain required for poly(ADP-ribose) synthesis (1). In living cells, activation occurs within seconds and leads to the covalent automodification of PARP with ADP-ribose polymers. In this reaction, the enzyme assumes a porcupine-like structure with up to 28 polymers of different sizes and structural complexities extending from the protein and a total molecular mass by far exceeding that of the 113-kDa PARP protein (for review, see Refs. 1 and 2). We have previously shown that PARP-bound polymers attract selected proteins from the vicinity of DNA strand breaks (3). The resulting protein–polymer complexes involved exceptionally strong noncovalent interactions (4).

The activation and up-regulation of p53 protein in mammalian cells is a late response to DNA break formation. The C-terminal domain of p53 binds to single- and double-stranded DNA ends, and this may be the first step in the activation of the transcriptional functions of p53 (for review, see Refs. 5 and 6). Neutralizing the ssDNA binding function of p53 with a peptide spanning the C-terminal domain leads to transactivation of the sequence-specific DNA binding domain in vitro (7). Microinjection of cells with an antibody specific for the C-terminal domain leads to transactivation of p53 even in the absence of induced DNA damage (8). Another feature in p53 activation is the stabilization and consequent up-regulation of the protein. Deletion studies revealed an essential degradation domain extending from amino acids 100 to 150 (9). Interestingly, E6-mediated degradation of p53 in mice could be blocked by an antibody specific for this region. This region resides within the binding site for SV-40 large T-antigen, which has also been shown to extend p53 half-life (10).

Recent cell biological studies suggest a functional association of PARP with p53 protein in mammalian cells. In rat cell lines constitutively expressing the temperature-sensitive p53135val, a transient colocalization of PARP and p53 was observed at the permissive temperature. Furthermore, a PAR-p53 complex could be reciprocally coprecipitated from these cells with anti-PARP or anti-p53 antibodies (11). Reciprocal coprecipitation of PAR-p53 complexes has also been demonstrated in human cells expressing wild-type p53 protein (12). Here we report that ADP-ribose polymers, either free or PARP-bound, target specific binding sites in p53 protein. Three polymer binding domains located in the core DNA binding and the oligomerization domains of p53 were identified. Polymer binding affected two major DNA binding functions of p53 protein, i.e. the sequence-specific binding to a p53 consensus sequence as well as the nonspecific binding to single strand DNA ends.

EXPERIMENTAL PROCEDURES

Materials

Polypeptides and Oligonucleotides—Human and murine p53 proteins were immunopurified from SF9 insect cells infected with recombinant baculoviruses as described previously (13) and were a generous gift of Dr. U. Hubscher (Department of Biochemistry, University of Zurich, Switzerland). The purified proteins migrated as a single band on SDS polyacrylamide gels. Anti-p53 antibody Pab421 was from Calbiochem. Histones and lysozyme were purchased from Sigma. Custom-designed synthetic oligopeptides were obtained from Anaqua Biomedical Services and Products, Wangen, Switzerland. Synthetic oligonucleotides were purchased from Microsynth (Switzerland). The following oligonucleotides were used: 5′-GACGAAATGCCCGCAGAAGCCCGCCCATAGGCTTTGTT-3′ (36-mer, N2) (14) and 5′-CAAGAAGGACATGCCGGCATTGTCCT-3′ (26-mer) and its complementary strand 5′-AGGACATGCCGGCATTGTCCTG3′ containing the palindromic consensus p53 target sequence (13, 15). Single-stranded oligonucleotides were 5′-end-labeled with [γ-32P]ATP and annealed to the complementary strand. Free or PARP-bound ADP-ribose polymers were synthesized with purified PARP (4). Free polymers were isolated by affinity chro-
matography on dihydroxyboronate resin. They had an average chain length of 16–20 residues, with ~6% of total ADP-ribose moieties being incorporated in branched polymeric chains (16).

**Methods**

**Poly(ADP-ribose) Blot Analysis**—After electrophoretic separation on SDS polyacrylamide gels (17), proteins were transferred onto nitrocellulose using a Bio-Rad dot blot apparatus. Proteins on nitrocellulose were gold-stained using the Protogold staining kit (British BioCell International), as recommended by the manufacturer. The blots were incubated with radiolabeled free or PARP-bound ADP-ribose polymers as described previously (18). Briefly, the nitrocellulose membrane was rinsed with three changes of Tri-buffered saline (TBS, 10 mM Tris, 0.15 M NaCl, pH 7.4) containing 0.05% (v/v) Tween 20 (TBST). Polymers of [32P]ADP-ribose (0.5 pmol/ml; 0.5 nmol total ADP-ribose; 0.5 nmol of total ADP-ribose) were dissolved in 10 ml of TBST and added to the nitrocellulose. After incubation for 1 h at room temperature, the membranes were extensively washed with TBST, dried, and subjected to autoradiography. For the incubations with PARP-bound polymers, nitrocellulose filters were blocked with 5% milk powder (in TBST) for 1 h at room temperature. 2 pmol of automated PARP were freshly prepared in a 100-μl reaction volume as described (4), diluted with 10 ml of TBST, and immediately used for incubations of the filters. The presence of residual DNA (60 ng/ml) and NAD+ (1.5 μmol) did not disturb polymer-protein interactions (3), and residual radiolabeled NAD+ did not bind to the blotted proteins, as determined in separate experiments (not shown).

**DNA Binding Assay**—Binding of p53 to ss- and dsDNA was assayed by electrophoretic mobility shift (19). Incubation of p53 with dsDNA oligonucleotide was carried out in 20 ml Hepes buffer, pH 7.9, containing 25 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.025% Nonidet P-40, 0.1 mg/ml bovine serum albumin, 10% glycerol, 0.2 ng of [32P]-labeled probe DNA, 10–40 ng of p53 protein in a final volume of 20 μl. Where indicated, sonicated herring sperm DNA (Sigma) was used as nonspecific competitor (1–3 μg/ml). The reaction mixture was incubated at room temperature for 45 min and subsequently loaded onto a native 4% polyacrylamide gel containing 0.5% TBE buffer (45 mM Tris borate, pH 8.3, 1 mM EDTA) and 0.05% Nonidet P-40. Samples were electrophoresed in 0.5% TBE at 200 V for 90 min at 4 °C and analyzed by autoradiography (19).

The binding of p53 to ssDNA was assayed by incubating the p53 protein (20–40 ng) in 40 ml Tris-HCl, pH 7.5, 1.5 mM diethiothreitol, 100 mM NaCl, 1 mM EDTA, and 20 mg/ml BSA in a final volume of 20 μl. The reaction was started by the addition of 0.3 ng (2 μl) of radiolabeled ssDNA. After incubation at 37 °C for 20 min, reaction products were analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel in 0.5% TBE buffer (25 mM Tris, 0.19 μM glycine, pH 8.5, 1 mM EDTA) followed by autoradiography of the dried gel.

**Effect of Poly(ADP-ribose) on p53-DNA Interactions**—The effect of poly(ADP-ribose) on the DNA binding activities of p53 was assayed by preincubating the p53 protein (40 ng) in the absence or presence of the indicated amount of [3H]poly(ADP-ribose) (160 dpm/μmol) for 15 min at room temperature in 20 μl of binding buffer. [3H]-Labeling was used for a precise quantification of the ADP-ribose polymers. The reaction was started by the addition of 0.1–0.3 ng (1–2 μl) of radiolabeled DNA, and the samples were analyzed by electrophoretic mobility shift assay. Where indicated, synthetic oligopeptides were present with poly(ADP-ribose) in the preincubation step.

**RESULTS**

The ability of p53 to bind ADP-ribose polymers was tested using a nitrocellulose polymer blot assay (18). Fig. 1A demonstrates the polymer binding to an electrophoretically pure preparation of murine p53. Fig. 1B shows a strong dot blot signal for poly(ADP-ribose) binding to a filter, as 0.75 pmol of murine or human p53 protein. This binding was comparable to that of histone H1, which like other members of the histone family, has a high affinity for poly(ADP-ribose) (4, 18). No binding was observed with proteinase K or DNase I (Fig. 1B).

In the experiment shown in Fig. 2, we determined whether PARP-bound polymers could also interact with p53 protein. A comparison of the binding of free polymers (Fig. 2A, lane 1) with that of PARP-bound polymers (Fig. 2B, lane 1) shows that the covalent linkage of polymers to the PARP enzyme does not impede interactions with p53 protein or histone H3, which served as a positive control. It should be noted that these experiments were performed under higher stringency conditions, i.e., the filters were washed twice with 1 M NaCl. This treatment removed between 20 and 25% of loosely bound polymers. Under identical conditions, there was no unspecific binding of the acidic polymers (either free or PARP-bound) to a highly basic protein, i.e., lysozyme, even when excessive amounts (350 pmol of lysozyme versus 10 pmol of p53) were present on the filters. Interestingly, histone H3, which like other members of the histone family (3, 4, 18) could be a natural competitor for polymer binding in vivo, did not significantly reduce polymer-p53 interactions. When free polymers (~70 pmol of polymers of an average size of 20 residues) were first incubated for 30 min with 300 pmol of histone H3 (Fig. 2A, lane 2) or when PARP-bound polymers (2 pmol of PARP) were preincubated with 900 pmol of histone H3 (Fig. 2B, lane 2) and then applied to filters containing 10 pmol of p53 protein, the dot blot signals were only slightly reduced. It should be noted that one molecule of histone H3 can “neutralize” two polymer molecules of an average size of 20 residues, as reported earlier (3). Strikingly similar results were obtained when the experiment was repeated with 10 pmol of histone H3 (instead of p53) blotted onto the filters. These data indicate that protein-bound...
polymers can readily exchange with suitable binding partners such as p53 or histone H3 but not with lysozyme, a protein that fails to bind to polymers in solution as well (4).

To determine the binding sites, a set of synthetic oligopeptides containing partially overlapping sequences of murine p53 protein was tested. Some of these peptides were framed to contain a previously identified polymer binding sequence motif that was identified in histone H3 using the peptide walking technique.2

Additional control experiments (not shown) confirmed our earlier report on the interactions of PARP-bound (ADP-ribose)-polymers (B) with free (A) or PARP-bound (ADP-ribose)-polymers (B) as described under "Methods." Peptide-bound radiolabeled ADP-ribose polymers were visualized by autoradiography. The peptide spanning amino acids positions 96–118 also tested negative (not shown). The black bars below the p53 domain map mark the sites of polymer binding.

*Fig. 3. Identification of poly(ADP-ribose) binding target sequences in murine p53 protein. Oligopeptides covering different regions of the p53 protein were tested in the polymer blot assay either with free (A) or PARP-bound (ADP-ribose)-polymers (B) as described under "Methods." Peptide-bound radiolabeled ADP-ribose polymers were visualized by autoradiography. The peptide spanning amino acids positions 96–118 also tested negative (not shown). The black bars below the p53 domain map mark the sites of polymer binding.*

The polymer complexes proved to be exceptionally resistant to pH partitioning, strong acids, chaotropes, detergents, and high salt concentrations (4). Furthermore, the binding could be localized to the tail regions of histones (18).

Heat-denatured p53 did not lead to complexes with the DNA probe and that the multiple shift bands representing p53-tetramer-DNA complexes and multiples thereof (Fig. 4; Refs. 21–23) could be converted into a single top-of-the-gel band after postincubation of p53-DNA complexes with monoclonal antibody PAb421. This antibody causes a supershift representing the largest complexes with reduced mobility (24).

Apart from the sequence-specific binding function located in the core domain, p53 binds to single- and double-stranded DNA ends (14, 25, 26). This function maps to the C-terminal domain of p53 protein and has been correlated to the ability of p53 to promote DNA renaturation and strand transfer (14, 25). A single-stranded radiolabeled oligonucleotide (36 nucleotides) was used in an electrophoretic mobility shift assay, and the effect of poly(ADP-ribose) on p53 binding to ssDNA ends was determined. The autoradiographs of the gels were scanned for quantification. The results of Fig. 5A demonstrate a dose-dependent inhibition of p53-ssDNA complex formation. Interestingly, maximal inhibition of p53 binding was observed at poly(ADP-ribose) concentrations ranging from 40 to 100 nM (i.e., 0.8–2 pmol in the reaction mixture), whereas sequence-specific DNA binding was only moderately affected (Fig. 5B). A comparison of the inhibition curves in Fig. 5 suggests different types of polymer interactions with the p53 core domains as compared with the C-terminal oligomerization domain. Additional control experiments (not shown) confirmed the specific involvement of the C-terminal domain of p53 in ssDNA binding, since monoclonal antibody PAb421 prevented complex formation as previously shown (14).

**DISCUSSION**

We have previously reported that ADP-ribose polymers bind noncovalently to selected proteins, particularly histones (3, 4, 18), and that this binding is far stronger than would be expected from simple electrostatic interactions (3). The histone-polymers complexes proved to be exceptionally resistant to phenol partitioning, strong acids, chaotropes, detergents, and high salt concentrations (4). Furthermore, the binding could be localized to the tail regions of histones (18).
In the present study, we have identified tumor suppressor protein p53, the key component of an important DNA damage signal pathway, as a target for poly(ADP-ribose) binding. The binding involves three domains of 23–26 amino acids mapping to important functional domains of p53 (Fig. 3). Poly(ADP-ribose) changes the DNA binding functions of p53 dramatically. We have calculated that a single polymer molecule of 20 ADP-ribose residues can block (or reverse) the sequence-specific binding of four molecules of p53 to its consensus DNA sequence (Figs. 4, 5), which requires the formation of a tetrameric complex. Likewise, p53 binding to single strand DNA ends was inhibited by poly(ADP-ribose) (Fig. 5). This highlights a molecular mechanism by which the polymers clustered on PARP at DNA breakage sites could affect the activation of p53 and/or interfere with its transcriptional function. Naturally, this interference could be blocked in vivo by other polymer-binding proteins, such as members of the histone family, which are more abundant in living cells than p53 protein. However, the results of Fig. 2 suggest that despite the formation of salt-stable polymer-protein complexes (for example with histone H3 or other histones (4, 18)), complexed polymers may readily exchange with suitable binding partners such as p53 protein and form new complexes. The possibility of a dynamic exchange of binding partners is a new feature of poly(ADP-ribose)-protein interactions that needs to be pursued in more detail.

Recent reports shed light on possible mechanisms by which poly(ADP-ribose) could play the role of an endogenous transactivator of p53 functions. Neutralizing the ssDNA binding function of p53 with a peptide spanning the C-terminal domain has been shown to transactivate the sequence-specific DNA-binding function in vitro (7). Microinjection of cells with an antibody specific for the C-terminal domain leads to transactivation of p53 even in the absence of induced DNA damage (8). The present study suggests that poly(ADP-ribose) could be a resident factor at DNA breakage sites, relaying transactivation of p53 by neutralizing the ssDNA binding C-terminal domain. Alternatively, poly(ADP-ribose) could block the transcriptional role of p53 by inhibiting its sequence-specific binding activity located in the core domain of the protein (5, 6). Another possibility is that poly(ADP-ribose) plays a role in p53 up-regulation by protecting the protein from proteolytic degradation. It is noteworthy that one polymer binding site (amino acids positions 153–178; Fig. 3) is located near a proteolytic cleavage site, suggesting that polymer binding may protect this sequence or make it conformationally unaccessible to proteolysis. Such protection has been observed following binding of SV-40 large T antigen to this region (10) or when monoclonal antibodies were bound adjacently to this region (9).

Several recent reports shed light on how poly(ADP-ribose) could be involved in p53 regulation in mammalian cells in vivo. Apart from the demonstration of a temporary colocalization of PARP and p53 protein in rodent cells, PARP-p53 complexes could be coprecipitated with anti-PARP and anti-p53 antibodies from rodent and human cells (11, 12). Another report (27) shows that mutant V79 cells, either deficient in poly(ADP-riboseylation) activity or with reduced NAD+ availability for poly(ADP-ribose) synthesis, are severely deficient in constitutive p53 expression and p53 up-regulation after treatment with etoposide, a topoisomerase II inhibitor. These cells were also deficient in downstream end points of p53 signaling, i.e. lack of activation of a reporter gene under the control of a p53 consensus binding sequence and no induction of apoptosis by etoposide (27). Consistent with these observations, embryonic fibroblasts from PARP-null mice were found to express very low constitutive levels of p53 protein and a 4–5-fold lower up-regulation of p53 protein in response to DNA damage as compared with PARP-proficient control cells (28). Taken together these data indicate that poly(ADP-ribose) plays a positive role in p53 activation and up-regulation. However, it is likely that the interrelationship between poly(ADP-ribose) and p53 is more complex. A recent report on the PARP-null cell phenotype suggests that poly(ADP-ribose) plays both an upstream as well as a collateral role in DNA damage signaling through p53 (28).

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