Regulation of p53 Sequence-specific DNA-binding by Covalent Poly(ADP-ribosyl)ation

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We have characterized the covalent poly(ADP-ribosyl)ation of p53 using an in vitro reconstituted system. We used recombinant wild type p53, recombinant poly(ADP-ribose) polymerase-1 (PARP-1) (EC 2.4.2.30), and βNAD⁺. Our results show that the covalent poly(ADP-ribosyl)ation of p53 is a time-dependent protein-poly(ADP-ribosyl)ation reaction and that the addition of this tumor suppressor protein to a PARP-1 automodification mixture stimulates total protein-poly(ADP-ribosyl)ation 3- to 4-fold. Electrophoretic analysis of the products synthesized indicated that short oligomers predominate early during hetero-poly(ADP-ribosyl)ation, whereas longer ADP-ribose chains are synthesized at later times of incubation. A more drastic effect in the complexity of the ADP-ribose chains generated was observed when the βNAD⁺ concentration was varied. As expected, increasing the βNAD⁺ concentration from low nanomolar to high micromolar levels resulted in the slower electrophoretic migration of the p53-(ADP-ribose)ₙ adducts. Increasing the concentration of p53 protein from low nanomolar (40 nM) to low micromolar (1.0 μM) yielded higher amounts of poly(ADP-ribosyl)ated p53 as well. Thus, the reaction was acceptor protein concentration-dependent. The hetero-poly(ADP-ribosyl)ation of p53 also showed that high concentrations of p53 specifically stimulated the automodification reaction of PARP-1. The covalent modification of p53 resulted in the inhibition of the binding ability of this transcription factor to its DNA consensus sequence as judged by electrophoretic mobility shift assays. In fact, controls carried out with calf thymus DNA, βNAD⁺, PARP-1, and automodified PARP-1 confirmed our conclusion that the covalent poly(ADP-ribosyl)ation of p53 results in the transcriptional inactivation of this tumor suppressor protein.

The covalent poly(ADP-ribosyl)ation of DNA-binding proteins in eucaryotes is a post-translational modification reaction that has been implicated in the modulation of chromatin structure and function in DNA-damaged and apoptotic cells (1–3). The immediate synthesis of poly(ADP-ribose) from βNAD⁺ in response to DNA strand break formation in vivo is mostly catalyzed by poly(ADP-ribose) polymerase-1 (PARP-1) (3/1–3). This enzyme was believed for some time to be the only nuclear DNA-dependent enzyme (EC 2.4.2.30) responsible for the synthesis of chromatin-bound ADP-ribose chains (3). However, over the last 4 years, and since the last International Symposium on protein-poly(ADP-ribosyl)ation (4), other novel and less abundant PARP-like proteins have been identified and reported (5–8).odule of ADP-ribose-polymerizing activity explains why PARP-1 (−/−) knockout cells still display a positive immunofluorescent nuclear signal when exposed to a fluorescently tagged monoclonal antibody specific for this unique nucleic acid (9). Nevertheless, it appears that about 90% of the total protein-bound polymers synthesized in DNA-damaged cells are assembled by PARP-1. Although most of these genotoxicity-dependent polymers of ADP-ribose seem to be covalently bound to PARP-1 itself (10–13), other chromatin proteins, including histones (14, 15), DNA-metabolizing enzymes (16, 17), and transcription factors (18–20), have been reported to be covalent targets for poly(ADP-ribosyl)ation. In this report, we have focused on the biochemical characterization of p53 poly(ADP-ribosyl)ation as well as the functional consequences of the substantial covalent modification of this tumor suppressor protein on its DNA-binding properties.

Tumor suppressor p53 is an inducible protein that accumulates in the nuclei following DNA damage. Interestingly, the increased expression of p53 has recently been shown to initially parallel the expression of PARP-1 in high grade lymphomas (21), and these events apparently precede the enzymatic activation of the latter in DNA-damaged cells (19). Not surprisingly, both p53 (22) and PARP-1 (23) have been “classified” as guardians of the eucaryotic genome. In fact, both DNA-binding proteins have been shown to physically associate via specific protein-protein interactions in genotoxicity treated cells (19, 24, 25). The molecular association between these DNA damage protein sensors may actually result in the βNAD⁺-dependent covalent poly(ADP-ribosyl)ation of the tumor suppressor protein both in vitro (19, 24) and in vivo (26). However, the functional consequences of the covalent poly(ADP-ribosyl)ation of p53 as a transcription factor remain to be elucidated.

It should be mentioned that the tumor suppressor p53 functions as a cell cycle checkpoint in maintaining genomic stability in mammals. In fact, over half of most human cancers contain mutations in the p53 tumor suppressor gene and over 90% of the p53 missense mutations are clustered within the sequence-specific DNA-binding domain. Overwhelming experimental evidence suggests that the functional inactivation of p53, especially its DNA-binding activity, is a crucial, and often obligatory step in the complex process of tumorigenesis. By contrast, p53 is either induced or activated in response to a plethora of stimuli, including DNA damage. The activation of p53 leads to one of two major cellular pathways: apoptosis or cell cycle arrest at the G1 phase preventing progression through the S phase of the cell cycle. Failing this response, the cell will undergo senescence, characterized by a G1 arrest, and cell death. These processes are mediated by the direct binding and activation of p53 in both mouse and human cells (27–30). The mechanism of action of p53 is dependent on the nature of the genotoxic (DNA-damaging) agent. In general, the p53 protein is activated by DNA damage, with a particular affinity for DNA double-strand breaks (31–34). This is achieved through the induction of p53-dependent transcriptional events, which may down-regulate the expression of those genes that mediate repair or DNA replication, or up-regulate those genes that mediate cell cycle arrest or apoptosis. This complex regulatory system is designed to ensure that DNA damage is repaired or cells are eliminated to prevent further transmission of genetic damage (35–38). Thus, p53 is a key element in the maintenance of genomic stability in eucaryotes.
phase until damaged DNA is repaired (27). Although the transcriptional properties of p53 are well established in cell cycle arrest following DNA damage, this is not necessarily so in p53-mediated apoptosis.

The tumor suppressor protein p53 may also function as a repressor of a variety of viral and cellular gene promoters that lack p53 binding sites (28–30), presumably via its carboxy-terminal fragment (31). Nevertheless, it is clear that the main function of this tumor suppressor protein is as a transcription factor in which p53 up-regulates specific cell cycle arrest-related genes (32, 33).

As a transcription factor, p53 recognizes a specific consensus DNA sequence consisting of two copies of the 10-bp motif, 5′-PuPuPuPuCAT/T/AA/GPyPyPy-3′, separated by a 0- to 13-bp spacer. Wild type p53 transactivates the expression of specific target genes by specifically binding to p53-binding sites in the sequences of these genes (32, 33). However, little is known about what kind of biochemical signals regulate the sequence-specific DNA binding affinity of p53. Because p53 has been shown to physically interact with PARP-1 (19) and ADP-ribose polymers (34) in DNA-damaged cells (see above), we have proceeded to accomplish the following goals. First, to biochemically characterize poly(ADP-ribosyl)ated p53 and second, to determine the ability of poly(ADP-ribosyl)ated p53 to bind to its consensus DNA sequence by electrophoretic mobility shift assays (EMSA). Our results clearly demonstrate the extensive poly(ADP-ribosylation) of wild type p53 as a function of: (i) the time of incubation, (ii) the βNAD+ substrate concentration, and (iii) the p53 protein concentration. Furthermore, we also demonstrate that poly(ADP-ribosyl)ated p53 does not efficiently bind to its consensus DNA sequence by EMSA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Electrophoresis molecular weight markers and reagents were purchased from Bio-Rad (Hercules, CA); [adenylate-32P]NAD (specific activity 500 Ci/mmol) was obtained from ICN Biomedicals (Costa Mesa, CA); T4 polynucleotide kinase was purchased from USB Corp. (Cleveland, OH); [γ-32P]ATP (specific activity 6000 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA); wild type recombinant human p53 Consensus Oligodeoxynucleotide Sequence—A 50-μl incubation reaction mixture containing T4 polynucleotide kinase buffer and unlabeled oligodeoxynucleotide was incubated with 200 pmol of [γ-32P]ATP in the presence of T4 polynucleotide kinase for 30 min at 37 °C. The product was purified by a series of precipitations and extractions with 330 mM ammonium acetate and 1% ice-cold ethanol. The final radioactivity of the product was 6 μCi/pmol as determined by scintillation counting.

**Poly(ADP-ribosyl)ation of wt-p53**—Triplicate samples containing the required concentrations of wt-p53 were incubated with 18 nM PARP-1 in the presence of 100 mM Tris HCl, pH 8.0, 10 mM MgCl2, 1 mM dithiothreitol as well as [β-32P]NAD+ and activated calf thymus DNA at 37 °C as indicated in the corresponding figure legend. 50 μl of SDS-loading buffer was added to one of each triplicate sample and loaded onto a 4–15% polyacrylamide gel. The poly(ADP-ribosyl)ated proteins were detected by autoradiographic analysis of the dried gel. Two samples from each triplicate were precipitated with 20% (v/v) trichloroacetic acid, washed, and counted to determine the total incorporation of ADP-ribose under each distinct set of conditions. One of the duplicate samples was used to determine the ADP-ribose polymer size distribution by reversed phase polyacrylamide gel electrophoresis (see below). A final concentration of 5 μM βNAD+ was used for the synthesis of unlabeled ADP-ribose polymers as necessary to analyze the effect of p53 poly(ADP-ribosyl)ation on DNA binding.

**Size Distribution of ADP-ribose Chains by High Resolution Polyacrylamide Gel Electrophoresis**—Acid-precipitable material was processed for analysis of the ADP-ribose chains as previously published (35). Briefly, the ADP-ribose polymers were chemically detached from protein acceptor with 0.1 N NaOH and 20 mM EDTA for 2 h at 60 °C, neutralized, and diluted in 60 mM tris borate-EDTA (TBE) buffer, pH 8.3. Protein-free ADP-ribose polymers were then subjected to electrophoresis on a (20 × 20 cm) 20% polyacrylamide gel. The size distribution of the ADP-ribose chains was visualized following overnight autoradiographic exposure to Kodak ( Biomax-MR) film.

**Determination of Wild Type p53 Sequence-specific DNA Binding by Electrophoretic Mobility Shift Assays**—The effect of the poly(ADP-ribosylation) of wt-p53 on the binding to its 125I-radioabeled consensus oligodeoxynucleotide sequence was determined by electrophoretic mobility shift assays (36) with the following modifications. First, either native or poly(ADP-ribosyl)ated p53 were incubated with 0.4 μg of γ-32P-radioabeled oligodeoxynucleotide probe in the presence of 100 mM Tris HCl, pH 8, 25 mM KCl, 0.025% Nonidet P-40, 0.1 mg/ml bovine serum albumin, 0.1 mM EDTA, and 5% glycerol for 15 min at 37 °C. Immediately following incubation, samples were loaded onto a native 4–20% acrylamide-TBE gradient gel and electrophoresed in TBE buffer at 150 V for ~60 min at 4 °C. Binding of wt-p53 to its consensus DNA sequence (20-mer) was analyzed by autoradiography following exposure of the dried gel to x-ray film.

**RESULTS**

Recently, we (19), as well as others (24–26), have shown the physical association of p53 with PARP-1 in DNA-damaged and apoptotic cells. However, this protein-protein association usually does not result in the covalent poly(ADP-ribosylation) of the tumor suppressor protein (19, 24, 25). In fact, the covalent poly(ADP-ribosylation) of p53 only takes place under specific conditions, e.g. when PARP-1 has not yet been proteolyzed by caspases 3 or 7 in apoptotic cells. That is why we could only confirm the covalent poly(ADP-ribosylation) of p53 in an apoptotic HeLa cell extract (19) after calf thymus PARP-1 was exogenously added. To gain further insight into the physiological significance of p53 poly(ADP-ribosylation) in DNA-damaged and apoptotic cells, we decided to biochemically characterize the enzymeology of this reaction and its consequences in the sequence-specific DNA binding properties of this tumor suppressor protein.

**The Covalent Poly(ADP-ribosylation) of Human wt-p53 is Time-dependent**—To confirm that wild type p53 is a covalent target for protein-poly(ADP-ribosylation), the tumor suppressor protein was incubated with 18 nM PARP-1, and 200 nM [β-32P]NAD+ under the conditions described under “Experimental Procedures.” Fig. 1A shows the amount of ADP-ribose incorporated as a function of the time of incubation from 0 to 120 min of incubation. We reproducibly observed that the level of protein-poly(ADP-ribosylation) was 3- to 4-fold higher in the presence of wt-p53 (circles) than in its absence (squares), suggesting that not only was PARP-1 efficiently auto-poly(ADP-ribosyl)ating but that the tumor suppressor protein was also covalently modified. To confirm the covalent association of ADP-ribose polymers with wt-p53, we carried out SDS-polyacrylamide gel electrophoresis through a 4–15% acrylamide gradient gel. Fig. 1B shows the Coomassie Blue-stained gel where the relative migration of wt-p53 (162 kDa) is clearly indicated to the left of the gel. Under these staining conditions, the 100 ng of PARP-1 (18 nM) utilized were not sufficient for strong staining. By contrast, Fig. 1C illustrates the signals developed upon autoradiographic exposure of the dried gel. Lanes 4–7 show the increase in p53 radiolabeling as the time of incubation was extended from 0 to 120 min. Fig. 1C also clearly shows that, although wt-p53 was clearly covalently radiola belled as a function of time, the efficiency of PARP-1-mediated modification also increased to the point where the intensity of the PARP-1 band significantly expanded as a result of hyper-poly(ADP-ribosylation). Therefore, it appeared as if the modification of wt-p53 increased the efficiency of PARP-1 automodification (see below).

Due to the dramatic change in the electrophoretic behavior of...
auto-poly(ADP-ribosyl)ated-PARP-1, we next decided to determine the size distribution of the protein-bound ADP-ribose polymers. We accomplished this by high resolution polyacrylamide gel electrophoresis. Samples were processed after chemical release from protein under alkaline conditions in the presence of EDTA (35). Fig. 1D shows the size distribution of the protein-free ADP-ribose chains synthesized when PARP-1 (18 nM) and wt-p53 (81 nM) were co-incubated as a function of time (see above). Lanes 1, 2, and 3 show the mono(ADP-ribo)syl)ation step of ADP-ribose polymer synthesis predominated in the first 5 min of incubation (AMP band). Also shown are ADP-ribose chains of up to 20 ADP-ribose units (lane 4) or more (lanes 5–7), which represent the polymers synthesized at 1 and 2 h of incubation. Lanes 8, 10, and 11 show the absence of protein-bound ADP-ribose polymers when DNA, βNAD+, or PARP-1 was omitted from the incubation mixture. By contrast, lane 9 shows the polymer size distribution of the enzyme products generated in the absence of wt-p53. Although the distribution of polymers was very similar without (lane 9) and with (lanes 5–7) wt-p53, the overall yield of (ADP-ribosyl)ated p53. Fig. 2A shows the significant decrease in the Coomassie Blue staining intensity of the wt-p53 (250 nM) band as the concentration of βNAD+ was increased from 0–1 μM (lanes 1–4) to 100–1000 μM (lanes 5–7). The remarkable decrease in staining intensity of this protein is apparently due to the increased levels of wt-p53 poly(ADP-ribose)lation at more physiological levels of βNAD+, namely 0.5–1.0 mM substrate concentration, because under these conditions, the protein adducts stay at the top of the gel. Our interpretation was later confirmed by autoradiographic analysis of the same gel upon exposure to x-ray film. As expected, Fig. 2B shows that the electrophoretic mobility of auto-poly(ADP-ribose)lated-PARP-1 significantly decreased from the typical 113-kDa position (lane 2) at 250 nM βNAD+, to the origin of the gel (lanes 4–7) at micromolar levels of βNAD+ (10, 100, 500, and 1000 μM, respectively). Therefore, we conclude that the molecules of wt-p53 that become covalently modified transform into heavily auto-poly(ADP-ribose)lated protein adducts that do not migrate into the gel, just like hyper-poly(ADP-ribose)lated-PARP-1.

To our knowledge, human wt-p53 (this report) and TFIIF (18), are the only transcription factors that have been carefully characterized in terms of the biochemistry of protein-poly(ADP-ribose)ylation. Thus, to evaluate the influence of all molecular components in the protein-poly(ADP-ribose)ylation mixture, we next determined the effect of p53 protein concentration on its poly-(ADP-ribose)ylation in the modification of this tumor suppressor protein.

The Covalent Poly(ADP-ribosyl)ation of Human wt-p53 Is Tumor Suppressor Protein Concentration-dependent—Fig. 3 illustrates the effect of p53 protein concentration on its poly-(ADP-ribose)ylation catalyzed by PARP-1 (18 nM) and βNAD+ (200 nM) after 30 min of incubation at 37 °C. Fig. 3A shows the Coomassie Blue-stained gel of this experiment as the wt-p53 protein concentration was increased from 0 to 1000 nM (lanes 1–7). Lanes 4 through 7 represent 160 nM, 250 nM, 500 nM, and 1.0 μM, respectively. These levels of p53 concentration were the...
only ones that contained enough protein for Coomassie Blue staining. Fig. 3B shows the autoradiograph of the same gel. We observed that, although a ratio of 2:1 of p53/PARP-1 was not enough for the ADP-ribose polymer modification of these polypeptides (Fig. 3B, lane 2), a ratio of at least 4:1 p53/PARP-1 was required for a positive signal (Fig. 3B, lane 3). Needless to say that higher concentrations of p53 (lanes 4–7) resulted in a stronger protein-poly(ADP-ribose)ylation signal. Interestingly, although the βNAD⁺ concentration remained constant (200 nM), the efficiency of the PARP-1 automodification reaction increased as the total amount of wt-p53 in the incubation reaction mixture was elevated (compare the thickness of the PARP-1 radiolabeled band on lanes 1 and 7).

Once we reproducibly confirmed the covalent poly(ADP-ribose)ylation of human wt-p53, we proceeded to determine its effect on the sequence-specific DNA binding of this tumor suppressor protein by EMSA.

Comparison of Sequence-specific DNA Binding between Native wt-p53 and Covalently Poly(ADP-ribose)ylated p53 by EMSA—Fig. 4 shows the EMSA analysis of both native p53 and poly(ADP-ribose)ylated p53 upon incubation with its [32P]-radiolabeled oligodeoxynucleotide consensus DNA sequence. Lane 1 displays the electrophoretic migration of the oligodeoxynucleotide probe alone. Lane 2 shows the mobility shift caused by the addition of 400 ng of wt-p53. Lanes 3, 4, and 5 display the effect of individual protein-poly(ADP-ribose)ylation reaction components on the wt-p53 DNA sequence-specific mobility shift. Although addition of βNAD⁺, the ADP-riboseylation substrate alone, did not affect the mobility shift signal (lane 3), addition of active calf thymus DNA significantly inhibited the mobility shift (lane 4). This was a fully anticipated result, because p53 is also known to possess nonspecific single-stranded DNA binding properties in its carboxyl-terminal domain (38–40). Therefore, we conclude that the result shown in Fig. 4, lane 4, is simply a competition effect for DNA binding. By contrast, the light inhibition observed upon addition of PARP-1 alone (lane 5) probably reflects the fact that PARP-1 itself is a DNA-binding protein that binds to DNA free ends, such as those present at 5’ and/or 3’ of the oligodeoxynucleotide probe. Presumably, the effect of PARP-1 inhibition is not quantitative, because it does not bind the probe efficiently, even at high nanogram levels (see Fig. 5 below). Surprisingly, addition of all protein-poly(ADP-ribose)ylation ingredients (Fig. 4, lane 6) caused the strongest inhibition of p53 sequence-specific DNA binding. Therefore, we conclude that the direct covalent poly(ADP-ribose)ylation of wt-p53, PARP-1, or both directly result in the specific inhibition of wt-p53 binding to its consensus DNA. To distinguish between these possibilities, we also carried out an incubation where active calf thymus DNA was omitted from the incubation mixture (Fig. 4, lane 7). Because omission of activating DNA from the EMSA incubation limits the extent of the automodification reaction of PARP-1, while still allowing the covalent poly(ADP-ribose)ylation of p53 (41), we could measure the effect of p53 poly(ADP-ribose)ylation on its sequence-specific DNA binding ability. Fig. 4, lane 7 shows that, under these conditions, there is a significant inhibition of p53 DNA binding (compare with lanes 2 and 6). Omission of βNAD⁺ alone (lane 8) significantly reduced the mobility shift of the p53 oligodeoxynucleotide probe as well. However, this effect was 1) mainly due to the presence of active calf thymus DNA and 2) to PARP-1 (compare with lanes 4 and 5). Finally, Fig. 5 shows a week interaction between PARP-1 and the radiolabeled DNA probe in the absence of calf thymus DNA (see above). In fact, addition of increasing amounts of PARP-1 from 7 to 70 ng did not result in an increased electrophoretic retardation of the DNA probe. Therefore, the addition of PARP-1 would not be expected to quantitatively inhibit the mobility shift caused by wtp53 (see Fig. 4, lane 5 above).
cycle arrest. Therefore, any molecular signal that turns
and often obligatory step to prevent the development of tumors.
Experimental evidence suggests that the functional activation of
\( p53 \)
may contain mutations in the
tumors, especially those of lung, colon, liver, and breast cancer,
or
the cell after substantial levels of DNA damage,
e.g.
\( 25 \text{ mM KCl}, 0.025\% \text{ Nonidet P-40, 0.1 mg/ml bovine serum albumin, 0.1}
\text{ mm EDTA, and 5\% glycerol for 15 min at 37}^\circ\text{C}. \)
Immediately following incubation, samples were loaded onto a native 4–20% acrylamide-TBE
gradient gel and electrophoresed in TBE buffer at 150 V for ~60 min at 4°C.
Lanes 1, DNA probe control; lanes 3 and 4, effect of either \( \beta\text{NAD}^- \)
or DNA on the \( p53 \) mobility shift, respectively; lane 5, effect of PARP-1 alone; lane 6, effect of the addition of PARP-1; and lanes 7 and 8, effect of DNA and \( \beta\text{NAD}^- \) omission in the DNA binding incubation mixtures, respectively.

DISCUSSION

The tumor suppressor protein \( p53 \) is thought to function as a
cell cycle checkpoint to maintain genomic stability in mam-
mals. As a result it may also be considered the “ultimate
gatekeeper.” A master molecule deciding the ultimate fate of
the cell after substantial levels of DNA damage, e.g.
“survival” or “cell death.” Not surprisingly, more than 50% of human
tumors, especially those of lung, colon, liver, and breast cancer,
may contain mutations in the \( p53 \) gene. In fact, over 90% of the
\( p53 \) missense mutations are typically clustered within its se-
quence-specific DNA-binding domain. Thus, overwhelming ex-
perimental evidence suggests that the functional activation of
\( p53 \), especially the DNA-binding activity of \( p53 \), is a crucial,
and often obligatory step to prevent the development of tumors.
The molecular activation of \( p53 \) may usually lead to one of two
major physiological pathways: programmed cell death or cell
cycle arrest. Therefore, any molecular signal that turns “on” or
“off” the DNA-binding properties of \( p53 \) either as a transcrip-
tion factor (32, 33) or as a genetic repressor (27–31) would be of
significant interest to study. Frequently, the physiological
function of crucial metabolic proteins is regulated by covalent
post-translational modification. Not surprisingly, Hupp et al.
(42) first proposed that the DNA-binding activity of \( p53 \) might be
regulated by the specific phosphorylation of the carboxy-
terminal DNA-binding domain. As we discussed above, it was
later found (39–40) that the carboxyl-terminal peptide of \( p53 \)
was specific for poly
targeting single-stranded DNA, which in turn
may be related to the potential function(s) of \( p53 \) as a gene
repressor (27–31) rather than as a transcription factor (32, 33).
Nevertheless, others (43) have reported that the phosphoryla-
tion of \( p53 \) at other peptide sites may lead to \( p53 \)-dependent transcriptional attenuation. However, it was not clear whether
the observed reduction in \( p53 \)-dependent transcriptional re-
sponses was due to the inability of this protein to oligomerize as
a tetramer (43). Indeed, tetrameric \( p53 \) has previously been
shown to be the latent and active form of this tumor suppressor
gene (44). Alternatively, the phosphorylation of the \( p53 \) car-
boxyl terminus might actually reduce the sequence-specific DNA binding of this transcription factor via conformational changes that may affect the ionic interactions of its central
domain with DNA itself. By contrast, the phosphorylation of
three serine residues at the amino terminus of \( p53 \) (serines 9, 18, and 37) was also reported to facilitate \( p53 \) transcriptional
function (45). However, the kinase modification of these sites
did not change the intracellular localization, oligomerization,
and DNA-binding properties of \( p53 \) (45). Therefore, it appears
that this type of post-translational modification of \( p53 \) is not
the direct post-translational mechanism to reversibly regulate
its transactivating properties. Recently, it was also demon-
strated that the sequence-specific DNA binding of this tumor
suppressor protein was activated by the acetylation of its car-
boxyl-terminal domain (46). Thus, it seems that both phospho-
rylation and acetylation target sites on \( p53 \) localized to either
the first 100 amino acids of its amino terminus or the last 90
amino acids of its carboxyl terminus (47–49).

Here, we concentrated on the biochemical characterization of
the covalent poly(ADP-ribosyl)ation of \( p53 \). The massive addi-
tion of ADP-ribose polymers of over 20 units (Fig. 1D)
immediately suggests that the addition of over 40 negative charges
two for every ADP-ribose unit) should result in the ionic
repulsion between post-translationally modified \( p53 \) and its
consensus sequence. In fact, the significant reduction in the
electrophoretic migration of poly(ADP-ribo-
syl)ated \( p53 \) adducts (Fig. 2) generated at physiological concentrations of \( \beta\text{NAD}^- \)
(e.g. 500–1000 \( \mu\text{M} \)) strongly suggested to us that the pro-
tein-protein interactions of \( p53 \) with PARP-1 (19, 24–26)
may result in the reversible inhibition of its sequence-specific DNA bind-
ing. Not surprisingly, we reproducibly observed that \( p53 \) lost
its DNA-binding properties upon covalent poly(ADP-ribo-
syl)ation (Fig. 4). We are currently in the process of identi-
fying the poly(ADP-ribose)ylation amino acid sites on both wild type
and mutant \( p53 \) molecules to determine whether they are ac-
tually localized on its centrally located sequence-specific DNA-
binding domain, its single-stranded DNA carboxyl-terminal
binding domain, or both. Our results are also in agreement
with the data of Malanga and Althaus (34) who demonstrated
that the presence of protein-free and highly branched polymers
of ADP-ribose in a \( p53 \) mobility shift assay resulted in the
strong inhibition of sequence-specific DNA binding. Although
ADP-ribose polymers are never protein-free in situ, proteins susceptible to covalent modification with highly branched polymers may be responsible for the reversible regulation of p53-dependent transactivation. Even though it has been clearly demonstrated that highly branched polymers are covalently bound to PARP-1 itself (10–12, 15–19, 35, 37) and not to histones (14–16), we report here for the first time, that highly complex ADP-ribose polymers can also be covalently bound to p53 (Fig. 2). Furthermore, we also show that the strong protein-protein association of p53 with PARP-1 in vitro (19) and in vivo (24–26) may lead to the higher efficiency of PARP-1 auto-modification shown in Figs. 1 and 3. Recently, we showed that the auto-modification reaction of PARP-1 directly activates the sequence-specific DNA binding of NF-κB (50), a well-established anti-apoptotic transcription factor. Therefore, low levels of DNA damage in cultured cells would presumably lead to the initial non-covalent recruitment of p53 (34) to DNA-damaged sites to allow for cell cycle arrest, DNA repair, and cell survival. By contrast, under high levels of DNA damage, the covalent poly(ADP-ribose)ylation of the tumor suppressor protein would inhibit cell cycle arrest and p53-dependent gene expression (27, 28). With the inhibition of the p53 sequence-specific DNA binding and the caspase-catalyzed degradation of PARP-1, severely DNA-damaged cells would initiate the execution phase of the cell death program (13). Finally, one should also keep in mind that, under specific conditions, NF-κB may actually facilitate programmed cell death and could be essential for p53-mediated apoptosis (51). Therefore, a better understanding of the interplay between NF-κB and p53 with regards to the activity of PARP-1 in programmed cell death needs to be evaluated in vivo as well.

Acknowledgments—We thank Dr. Hanswalter Zentgraf, Institute for Tumor Virology, German Cancer Research Center, Heidelberg, Germany for his gift of the baculovirus and E. coli recombinant p53 proteins used in some of the control experiments in this study.

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Regulation of p53 Sequence-specific DNA-binding by Covalent Poly(ADP-ribosyl)ation
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doi: 10.1074/jbc.M105215200 originally published online July 26, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105215200

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