The Sequence-specific DNA Binding of NF-kB Is Reversibly Regulated by the Autamodification Reaction of Poly (ADP-ribose) Polymerase 1*

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Recent studies suggest that the synthesis of protein-bound ADP-ribose polymers catalyzed by poly(ADP-ribose) polymerase-1 (PARP-1) regulates eucaryotic gene expression, including the NF-kB-dependent pathway. Here, we report the molecular mechanism by which PARP-1 activates the sequence-specific binding of NF-kB to its oligodeoxynucleotide. We co-incubated pure recombinant human PARP-1 and the p50 subunit of NF-kB (NF-kB-p50) in the presence or absence of βNAD⁺ in vitro. Electrophoretic mobility shift assays showed that, when PARP-1 was present, NF-kB-p50 DNA binding was dependent on the presence of βNAD⁺. DNA binding by NF-kB-p50 was not efficient in the absence of βNAD⁺. In fact, the binding was not efficient in the presence of 3-aminobenzamide (3-AB) either. Thus, we conclude that NF-kB-p50 DNA binding is protein-poly(ADP-ribose)ylation dependent. Co-immunoprecipitation and immunoblot analysis revealed that PARP-1 physically interacts with NF-kB-p50 with high specificity in the absence of βNAD⁺. Because NF-kB-p50 was not an efficient covalent target for poly(ADP-ribose)ylation, our results are consistent with the conclusion that the auto-poly(ADP-ribose)ylation reaction catalyzed by PARP-1 facilitates the binding of NF-kB-p50 to its DNA by inhibiting the specific protein-protein interactions between NF-kB-p50 and PARP-1. We also report the activation of NF-kB DNA binding by the autamodification reaction of PARP-1 in cultured HeLa cells following exposure to H₂O₂. In these experiments, preincubation of HeLa cells with 3-AB, prior to oxidative damage, strongly inhibited NF-kB activation in vivo as well.

Protein poly(ADP-ribose)ylation is a post-translational modification of DNA binding proteins in eucaryotes in vivo (1, 2). The acute synthesis of poly(ADP-ribose) from βNAD⁺ in response to DNA strand break formation is mostly catalyzed by poly(ADP-ribose) polymerase-1 (PARP-1) (1, 2). PARP-1 is an abundant constitutively expressed nuclear enzyme (3, 4). It is also a phylogenetically ancient protein widely conserved in eucaryotes, with a noticeable exception in yeast (2). The modular structure of PARP-1 indicates that this protein contains bipartite zinc fingers (5) in its N-terminal sequence. When bound to DNA nicks, the zinc finger motifs activate the C-terminal catalytic domain of PARP-1 to processively transfer the ADP-ribose moiety from βNAD⁺ to covalently modify acceptor proteins (5, 6). This dynamic synthesis and rapid clearance (7) of protein-bound (ADP-ribose) polymers has been implicated in eucaryotic DNA repair, DNA replication, and transcription. During this process, numerous nuclear proteins, including histones, DNA polymerases and ligases, Ca²⁺/Mg²⁺-dependent endonuclease, and transcription factors (e.g. TF₁F, YY1, and p53) are covalently poly(ADP-ribosyl)ated in vitro and/or in vivo (8–13). Furthermore, PARP-1 appears to also be involved in cellular commitment to apoptosis, because the proteolytic cleavage of PARP-1 by caspases 3 and/or 7 is frequently used as a hallmark of apoptotic execution (14, 15). In fact, it has been suggested that the cleavage of PARP-1 allows cells to conserve energy reserves (βNAD⁺ and ATP) by inactivating the ADP-ribose polymerizing activity of PARP-1 (15).

Until recently, most studies on PARP-1 had focused on its enzymatic activity following DNA damage (1, 2). However, it has also been suggested that PARP-1 may play a more sophisticated molecular role in chromatin structure and function by forming protein complexes with other proteins. For example, PARP-1 physically associates with DNA polymerase α and stimulates DNA replication in vitro without degrading βNAD⁺ (16). In addition, PARP-1 enhances activator-dependent transcription as an active component of the pre-initiation complex in vitro, and this enhancement appears to be silenced by its auto-poly(ADP-ribose)ylation (17). Roeder and co-workers (18) previously concluded that transcription factor TF₁C, a protein that stimulated nick translation, was identical to PARP-1. More recently, PARP-1 has also been shown to bind the oncogenic protein B-MYB to enhance its transactivating property (19). Therefore, PARP-1 may regulate the expression of specific genes by physical association with specific transcription factors. A good example for PARP-1-regulated gene expression events may be execution of the cell death program (15). Because the degradation of PARP-1 triggers the execution phase of apoptosis (14) and NF-kB is considered an anti-apoptotic transcription factor, we hypothesized that the nuclear activation of NF-kB might also be regulated by PARP-1.

Transcription factor NF-kB was originally described in B-lymphoid cells (20). Classic NF-kB is a heterodimer composed of a DNA-binding p50 subunit and a transactivating p65 subunit (RelA). NF-kB is pre-synthesized in the cytosol and immediately sequestered as a protein complex with IκB in the cyto-

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†The abbreviations used are: PARP-1, poly(ADP-ribose) polymerase-1; TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; EMSA, electrophoretic mobility shift assay; 3-AB, 3-aminobenzamide; PMSF, phenylmethylsulfonyl fluoride; LZ, leucine zipper; IUR, immediate up-stream region.
plasm (21). NF-κB is activated for nuclear translocation by specific extracellular stimuli. In fact, this phenomenon has been shown to be independent of protein synthesis (22). A plethora of heterogeneous, seemingly unrelated signal molecules, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), lipopolysaccharide, γ-radiation, etoposide, or H2O2 can activate NF-κB in target cells. These stimuli activate a signal transduction cascade that targets IkB degradation in the cytoplasm. As a result of this process NF-κB reveals its nuclear localization signal and translocates to the nucleus. The rapidly nuclear-translocated NF-κB activates genes concerned with inflammatory or immune responses such as inducible nitric-oxide synthase, IL-1, β, IL-6, and TNF-α. Other studies have shown that NF-κB activation is accompanied by the intracellular generation of reactive oxygen species (23–26). For example, the addition of micromolar concentrations of H2O2 can also activate NF-κB in Jurkat and HeLa cells, a process that may be blocked in the presence of antioxidants (23, 25). The notion that H2O2 may specifically lead to NF-κB nuclear targeting is noteworthy, because H2O2 can also activate the protein-poly(ADP-ribose)ylation pathway and automodification reaction of PARP-1 by causing DNA strand breaks (27–29).

As indicated above, eukaryotic gene expression may be controlled by the physical association of specific transcription factors with other proteins, such as PARP-1, which together form a multiprotein complex on enhancers and promoters (30). For example, studies have recently suggested that PARP-1 participates in the regulation of eukaryotic transcription and gene expression, including NF-κB (17, 19, 31–33). In fact, PARP-1 was shown to be required for proper NF-κB activation in lipopolysaccharide-treated mice (33). Furthermore, the NF-κB activation-dependent transcription of nitric-oxide synthase was suppressed by PARP-1 inhibitors in murine macrophages (31). However, the exact biochemical mechanism that mediated the PARP-1-dependent transcriptional activation was not shown. Therefore, to determine the biochemical role of PARP-1 in NF-κB activation in vitro, we co-incubated pure PARP-1 and the p50 subunit of NF-κB (NF-κB-p50) in the presence or absence of βNAD+. Our study illustrates the βNAD+–dependent binding of NF-κB-p50 to its oligodeoxynucleotide, a reduction in the DNA binding efficiency by 3-AB, and the physical interaction of PARP-1 with NF-κB-p50. Furthermore, our study also demonstrates a strong relationship between protein-poly(ADP-ribose)ylation and NF-κB activation in oxidatively stressed HeLa cells.

EXPERIMENTAL PROCEDURES

Purification of Human PARP-1—Construction of recombinant baculovirus containing cDNA of human PARP-1, its expression in Spodoptera frugiperda, and protein purification are described elsewhere (34). Poly(ADP-ribose)ylation in Vitro—For Fig. 4B (see below), human PARP-1 and human NF-κB-p50 (Promega) were incubated for 20 min at room temperature in a mixture (20 μl) containing 100 μM Tris-Cl (pH 7.8), 10 mM MgCl2, 1 mM dithiothreitol, and 20 μM/ml synthetic octamer DNA (5′-GGAATTCC-3′, Integrated DNA Technologies) (35). For this experiment, we used 1.5 ng of βNAD+ (ICN) as a substrate. The reaction was terminated by adding 2× SDS sample buffer, and proteins were fractionated through a 4–15% gradient polyacrylamide gel. Poly-(ADP-riboseylated proteins were visualized by autoradiography.

For Fig. 4A, pure PARP-1 and NF-κB-p50 were incubated for 20 min at room temperature in a mixture (20 μl) containing 20 μM Tris-Cl (pH 8.0), 60 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.05% Nonidet P-40, 10% glycerol, and 50 μg/ml bovine serum albumin (12). In this case, 1.5 ng of βNAD+ and 1.5 ng of NF-κB-oligodeoxynucleotide (2.5 ng of DNA) was then added, and the mixtures were further incubated for another 20 min. After terminating the reaction with 2× SDS sample buffer, proteins were fractionated by SDS-PAGE through a 4 to 15% gradient gel.

Electrophoretic Mobility Shift Assay (EMSA)—For the radiolabeled probe of NF-κB, a duplex oligodeoxynucleotide containing the consensus sequence (5′-AGTTGAGGGACCTTTCCCAGGC-3′, Santa Cruz Biotechnology) was end-labeled with [γ-32P]ATP (ICN) and T4 DNA polymerase (United States Biochemical Corp.). For EMSA with purified proteins, pure PARP-1 and NF-κB-p50 were incubated for 20 min at room temperature in a binding buffer containing 20 μM Tris-Cl (pH 8.0), 60 mM KCl, 5 mM MgCl2, 0.05% Nonidet P-40, 10% glycerol, and 50 μg/ml bovine serum albumin (12). In some reactions, βNAD+ (Roche Molecular Biochemicals), 3-aminobenzoamide (3-AB) (Sigma Chemical Co.), or duplex octamer DNA (5′-GGAATTCC-3′) was included. Equal amounts of βP-labeled NF-κB-oligodeoxynucleotide (2.5 ng) were added, and the mixtures (20 μl) were incubated for another 20 min. Samples were separated through a native 5% polyacrylamide gel containing 17.8 mM Tris borate and 0.4 mM EDTA. Protein-oligodeoxynucleotide complexes were visualized by autoradiography. For EMSA with HeLa nuclear extracts, cell treatment and nuclear extract preparation were done immediately before EMSA. Extracts containing 10 μg of protein each, 1 μg of poly(dI-dC), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) were incubated at room temperature for 10 min in the binding buffer before βP-labeled NF-κB oligodeoxynucleotide (1.5 ng) were added. Reactions (20 μl) were incubated for another 15 min, and samples were electrophoresed through a native 5% polyacrylamide gel containing 45 mM Tris borate and 1 mM EDTA. For the identification of protein-oligodeoxynucleotide complex, 1 μg each of NF-κB-p50 antibody (Santa Cruz Biotechnology) or control antibody (PAb421 for p53; Oncogene Research) was incubated with nuclear extracts for 30 min at 4 °C before poly(dI-dC) and βP-labeled NF-κB-oligodeoxynucleotide were added.

In the competition experiment shown in Fig. 1 below, we determined the off-rate for NF-κB-p50 from its DNA probe by EMSA also. To accomplish this, we incubated 80 ng each of NF-κB-p50 with pure βP-labeled NF-κB-oligodeoxynucleotide (Promega) in the presence of increasing amounts of unlabeled oligodeoxynucleotide probe for 20 min at room temperature. Co-immunoprecipitation/Immunoblotting—Equimolar amounts of PARP-1 (800 ng) and NF-κB-p50 (350 ng) were incubated for 30 min at 4 °C in a buffer containing 10 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 0.1% Nonidet P-40 (19). As a control experiment, NF-κB-p50 (350 ng) alone was also incubated. Protein was immunoprecipitated for 1 h at 4 °C with goat polyclonal antibody (Santa Cruz Biotechnology). The immune complexes were pull-downed by adding 30 μl of protein G-agarose beads (1:1 slurry) and incubating for 45 min at 4 °C with rocking. Beads were washed five times with the same buffer and adding 2× SDS sample buffer and boiling for 5 min eluted the bead-bound proteins which were fractionated by SDS-PAGE and gel under non-reducing conditions, and proteins were electrotransferred to a polyvinylidene difluoride membrane. The membrane was immuno-blotted with rabbit anti-NF-κB-p50 polyclonal antibody (Santa Cruz Biotechnology) and NF-κB-p50 was detected with horseradish peroxydase-conjugated anti-rabbit IgG antibody (Sigma) and an ECL chemiluminescence kit (Amersham Pharmacia Biotech).

Cell Culture and Treatment—Human cervical adenocarcinoma cell line HeLa (CCL-2; American Type Culture Collection) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in humidified 5% CO2 and air. For H2O2 and 3-AB treatment, exponentially growing cells were seeded in disc plates (≈0.4 × 106 cells/60-mm-diameter disc) 20–24 h before treatment. Cells were pre-treated with 10 mM 3-AB or its vehicle control for 1 h before treatment with H2O2 for 1 h. H2O2 was diluted from 30% stock (Sigma) immediately before use. The vehicle did not interfere with NF-κB DNA binding (data not shown).

Nuclear Extract Preparation—Extracts were prepared by a modified method from Dim Gap et al. (36) immediately before EMSA. Treated cells were washed with phosphate-buffered saline, harvested to microcentrifuge tubes, and briefly centrifuged (16,000 × g, 4 °C, 15 s). Cells were washed again with ice-cold phosphate-buffered saline, pelleted, and resuspended at 4 °C in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, and protease inhibitors (5 μg each of aprotinin, leupeptin, and pepstatin per ml). Cells were allowed to swell on ice for 15 min, then centrifuged at 40°C (0.15% final conc.) was added, and each sample was vigorously mixed. Nuclei were pelleted (16,000 × g, 4 °C, 30 s) and resuspended in a buffer containing 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, and protease inhibitors (5 μg each of aprotinin, leupeptin, and pepstatin per milliliter). Nuclear lysates were maintained on ice for 15 min with occasional mixing. Nuclear extracts were cleared (16,000 × g, 4 °C, and
PARP-1 Regulates the Sequence-specific DNA Binding of NF-κB

Fig. 1. Specificity of NF-κB DNA binding and determination of the off-rate by competition of increasing amounts of unlabeled DNA probe with a fixed amount of its 32P-labeled oligodeoxynucleotide consensus sequence. EMSA was performed as described under “Experimental Procedures.” 80 ng of NF-κB-p50 was incubated with ~1 × 10⁵ cpm of 32P-labeled duplex NF-κB oligodeoxynucleotide (0 pmol, lane 1; 0.18 pmol, lane 2; 0.9 pmol, lane 3; 1.8 pmol, lane 4; 5.4 pmol, lane 5; 9.0 pmol, lane 6; 12.6 pmol, lane 7; and 18 pmol, lane 8) in a 20-μl incubation mixture at room temperature. After 20 min, the samples were fractionated through a 5% native polyacrylamide gel containing 0.2× Tris borate EDTA buffer. The NF-κB-p50 oligodeoxynucleotide complexes were visualized by autoradiography.

5 min) and transferred to new tubes. For 3-AB pretreated cells, 10 μM of the inhibitor was included throughout the preparation of nuclear extracts. Protein concentrations were determined by Bradford assay.

RESULTS

To be able to determine the molecular role of protein-poly(ADP-ribosyl)ation in the sequence specific binding of NF-κB-p50 to its consensus DNA sequence, we first proceeded to show the specificity of DNA binding of this polypeptide to its 32P-radiolabeled DNA probe by electrophoretic mobility shift assays (EMSA).

Fig. 1A shows the off-rate of NF-κB-p50 from its radiolabeled probe following the addition of increasing amounts of unlabeled DNA probe to a fixed amount of its 32P-labeled oligodeoxynucleotide consensus sequence, and 80 ng of the DNA binding protein. After 20 min of incubation, the samples were fractionated through a 5% native polyacrylamide gel containing 0.2× Tris borate EDTA buffer and the protein-oligodeoxynucleotide complexes were visualized by autoradiography. As Fig. 1A (lanes 1–8) shows, the mobility shift of the radiolabeled probe disappeared as a function of the amount of unlabeled oligodeoxynucleotide added. Therefore, from this experiment we conclude that our mobility shift test can be applied to study the role of protein-poly(ADP-ribosyl)ation in the sequence-specific DNA binding of NF-κB-p50. Fig. 1B shows the disappearance of the mobility shift as a function of the concentration of unlabeled DNA added to the EMSA mixture and densitometric analysis of the data illustrated in Fig. 1A. From the graphical representation observed here, it was clear that 50% of DNA binding specificity was lost when less than 1 pmol of unlabeled DNA probe was added, even after 20 min of incubation. These results are consistent with a strong and highly specific binding of pure NF-κB-p50 to the radiolabeled DNA probe used in these studies (see above under “Experimental Procedures”).

Effect of Poly(ADP-ribosyl)ation on NF-κB-p50 DNA Binding—Previous studies have suggested that PARP-1 may participate in NF-κB activation in various cell lines (31–33, 37). To examine the effect of protein-poly(ADP-ribosyl)ation on NF-κB-p50 DNA binding in vitro, we co-incubated pure PARP-1 and NF-κB-p50 either in the presence or absence of βNAD⁺. The enzymatic activity of PARP-1 was allowed to proceed for 20 min at room temperature, and the 32P-labeled oligodeoxynucleotide containing the consensus sequence for NF-κB was added. To avoid a potential effect of exogenous DNA on NF-κB-p50 DNA binding, nicked DNA was omitted as an enzymatic activator of NF-κB-p50 DNA binding was reversed by competition of increasing amounts of unlabeled DNA binding specificity was lost when less than 1 pmol of unlabeled DNA probe.
NF-κB-p50, the automodification reaction of PARP-1, or both. Those NF-κB-p50–oligodeoxynucleotide complexes (Figs. 2, lanes 6–8) co-migrated with that of the NF-κB-p50 control (Fig. 2, lane 2), suggesting that NF-κB-p50 DNA binding became independent of PARP-1. Regardless of the PARP-1 concentration in the incubation mixture, the radiographic intensity of the NF-κB-p50–oligodeoxynucleotide complex (lanes 6–8) was similar to that of the NF-κB-p50 control (Fig. 2, lane 2). Therefore, these data suggest that there was no dilution effect of PARP-1 to NF-κB-p50 DNA binding.

3-Aminobenzamide (3-AB) is a well-established competitive inhibitor of βNAD⁺ in the protein-poly(ADP-ribosyl)ation reaction catalyzed by PARP-1. Therefore, we next evaluated the efficiency of the βNAD⁺-dependent NF-κB-p50 DNA binding following co-incubation of PARP-1, NF-κB-p50, and βNAD⁺ in the presence of 10 mM 3-AB (see above).

Inhibition of NF-κB-p50 DNA Binding by PARP-1 in the Presence of βNAD⁺ and 3-AB—Fig. 3 shows that addition of 400 ng of PARP-1 inhibited NF-κB-p50 DNA binding in the absence of βNAD⁺. This inhibition was accompanied by an apparent supershift of radiolabeled oligodeoxynucleotide (Fig. 3, lane 3), as compared with NF-κB-p50 control (80 ng; Fig. 3, lane 2). As shown above, addition of 200 μM βNAD⁺ resulted in the nullification of the PARP-1 inhibitory effect and NF-κB-p50–oligodeoxynucleotide complex co-migrated with that of the NF-κB-p50 control (Fig. 3, lane 4). By contrast, inhibition of the auto-poly(ADP-ribosyl)ation reaction of PARP-1 with 10 mM 3-AB led to the characteristic inhibition of NF-κB-p50 DNA binding by native PARP-1 (Fig. 3, lane 5). These data demonstrate that the βNAD⁺-dependent NF-κB-p50 DNA binding is indeed the result of covalent protein-poly(ADP-ribosyl)ation.

Overall, data shown in Figs. 2 and 3 indicate that PARP-1 interacts with NF-κB-p50 when PARP-1 is not poly(ADP-ribosyl)ated. Thus, when NF-κB-p50 interacts with PARP-1, it does not efficiently bind to its oligodeoxynucleotide. However, the auto-poly(ADP-ribosyl)ation of PARP-1 does not allow protein-protein interactions with NF-κB-p50, which in turn facilitates the DNA sequence-specific binding of the latter.

We next proceeded to determine whether this effect observed on NF-κB-p50 DNA binding, as a result of the addition of PARP-1 and βNAD⁺ (Figs. 2 and 3), was due to the poly(ADP-ribosyl)ation of the transcription factor or the automodification reaction of PARP-1.

Identification of the Covalent Poly(ADP-ribose) Protein Acceptor with the NF-κB-p50 Oligodeoxynucleotide as Activating DNA—The protein-poly(ADP-ribosyl)ation assay was carried out under the same conditions shown in Fig. 2, except that [32P]βNAD⁺ (142.5 mCi/mmol) was used to visualize poly(ADP-ribosyl)ation acceptors by autoradiography and the 32P-radio-labeled oligodeoxynucleotide was replaced with unlabeled DNA probe. We observed that PARP-1 was extensively poly(ADP-ribosyl)ated (Fig. 4A) both in the absence or presence of NF-κB-p50. On the other hand, NF-κB-p50, whose native molecular mass is ~50 kDa, was not susceptible to covalent poly(ADP-ribosyl)ation as indicated by the absence of a radio-labeled protein at or above the 45.7-kDa electrophoretic mark.

The lack of NF-κB-p50 covalent poly(ADP-ribosyl)ation was observed with three different concentrations of PARP-1 (Fig. 4A, lanes 2–4). Therefore, our data suggest that the auto-poly(ADP-ribosyl)ation of PARP-1, not the heteromodification of NF-κB-p50 was responsible for activating the βNAD⁺-dependent NF-κB-p50 DNA binding when PARP-1 and NF-κB-p50.

![Diagram](image-url)
plexes were visualized by autoradiography. Lane 1 and lane 2, NF-κB-p50, PARP-1 was efficiently auto-poly(ADP-ribosyl)ation, both in the presence and absence of NAD⁺ (35). The mixture was incubated at the antibody at 4 °C for 30 min at 4 °C before a goat anti-PARP-1-specific polyclonal antibody was added. Subsequently, the mixture was incubated with the antibody at 4 °C for 60 min more. Protein G-agarose beads were added next, and the samples were further incubated for 45 min at 4 °C with rocking. The beads were then washed five times, and bead-bound proteins were eluted by adding 2× SDS sample buffer and by boiling for 5 min. Proteins were fractionated by SDS-PAGE on an 8% gel under non-reducing conditions and electrotransferred to polyvinylidene difluoride membrane afterward. The membrane was immunoblotted with a rabbit anti-NF-κB-p50-specific polyclonal antibody, and protein was detected with the horseradish peroxidase-conjugated anti-rabbit IgG antibody and ECL chemiluminescence kit. Representative data from three independent experiments are shown.

**Fig. 5.** Effect of random DNA on the binding of NF-κB-p50 to its oligodeoxynucleotide. EMSA was carried out as described under "Experimental Procedures." PARP-1 (0 (–) and 800 (+) ng) and NF-κB-p50 (0 (–) and 80 ng) were incubated in the presence of increasing amounts of octameric duplex DNA (5'-GGAATTCC-3') at a concentration of 0 (–), 0.5 (–), and 10 (++) µg/ml, respectively, with (+) or without (-) 200 µM βNAD⁺. The mixture was incubated at room temperature for 20 min, and 1.0 µl of 32P-labeled NF-κB oligodeoxynucleotide (~2.5 ng) was added next. The 20 µl reaction mixture was incubated for another 20 min, and the samples were separated at room temperature through a native 5% polyacrylamide gel containing 17.8 mM Tris borate and 0.4 mM EDTA. Protein-oligodeoxynucleotide complexes were visualized by autoradiography. Lane 1, PARP-1 control; lane 2, NF-κB-p50 control.

NF-κB-p50 were co-incubated with the consensus sequence DNA oligodeoxynucleotide.

Next, to confirm the inability of NF-κB-p50 to act as a poly(ADP-ribosyl)ation acceptor specificity by mixing pure NF-κB-p50, PARP-1, β2P-labeled βNAD⁺, and oligomeric DNA as an enzymatic activator. Autoradiographic analysis of these incubations showed that NF-κB-p50, whose native molecular mass is about 50 kDa, was not susceptible to covalent poly(ADP-ribosyl)ation under these conditions either (Fig. 4B, lanes 4–8). Again, the insensitivity of NF-κB-p50 to covalent poly(ADP-ribosyl)ation was reproducible at three different βNAD⁺ concentrations (0.5, 20, and 800 µM) (Fig. 4B, lanes 6, 7, and 8, respectively). By contrast, PARP-1 was efficiently auto-poly(ADP-ribosyl)ated, both in the presence or absence of NF-κB-p50 (Fig. 4B, lanes 2, 3, and 5–8). As expected (6), the electrophoretic mobility of auto-activated PARP-1 changed with the substrate concentration. For example, in the presence of high micromolar concentrations of βNAD⁺, which are close to or exceed the Km of PARP-1 for βNAD⁺ (~50 µM), PARP-1 was extensively auto-poly(ADP-ribosyl)ated (Fig. 4B, lanes 2, 5, 7, and 8). Under these conditions, the radiolabeled protein stayed at or near the top of the gel. In summary, these data confirm that the βNAD⁺ dependent restoration of NF-κB-p50 DNA binding shown in Fig. 2 was not the result of the covalent poly(ADP-ribosyl)ation of NF-κB-p50 itself but rather, the result of the auto-poly(ADP-ribosyl)ation reaction catalyzed by PARP-1.

Protein-bound ADP-ribose polymers are regarded as a third type of nucleic acids (2). Therefore, it is feasible to suggest that random DNA may also exert a stimulatory effect on the binding of NF-κB-p50 to its oligodeoxynucleotide. Thus, we next conducted EMSA by adding duplex octameric DNA (5'-GGAATTCC-3') to the incubation mixtures, in the range of 0.5 to 10 µg/ml, and in the absence of βNAD⁺ (35).

**Effect of Random DNA on the Binding of NF-κB-p50 to Its Oligodeoxynucleotide in the Absence of βNAD⁺** —In the absence of PARP-1 automodification, e.g. no pyridine dinucleotide, the synthetic octameric duplex DNA facilitated the binding of NF-κB-p50 to its oligodeoxynucleotide in a concentration-dependent manner (Fig. 5, lanes 4–6). In fact, when compared with the control (Fig. 5, lane 4), 0.5 µg/ml of this octameric DNA partially re-activated the NF-κB-p50 DNA binding (Fig. 5, lane 5), and this activation was complete with 10 µg/ml of the DNA (Fig. 5, lane 6). It should be noted that a similar DNA-dependent binding of NF-κB-p50 to its oligodeoxynucleotide without protein-poly(ADP-ribosyl)ation was also observed with other types of random DNA additions, including sonicated DNA from Escherichia coli or DNase I-treated DNA from calf thymus (data not shown). Therefore, these data suggest that deoxyribonucleic acid in general is functionally similar to ADP-ribose polymers in overcoming the macromolecular association of PARP-1 with NF-κB-p50. Next, to demonstrate the specificity of the PARP-1-NF-κB-p50 complex formation, we carried out co-immunoprecipitation experiments.

**Co-immunoprecipitation of NF-κB-p50 with PARP-1-specific Antibody**—Because Figs. 2–5 consistently suggested the formation of a highly specific PARP-1-NF-κB-p50 macromolecular complex in the absence of enzymatic automodification conditions, we decided to test whether PARP-1 was able to physically associate with NF-κB-p50 by performing co-immunoprecipitation and reciprocal Western blot analysis. Mixtures containing equimolar amounts of PARP-1 (800 ng) and NF-κB-p50 (350 ng) were immunoprecipitated with an anti-PARP-1 polyclonal antibody, and immune complexes were subsequently pulled down with protein G-agarose beads as indicated under "Experimental Procedures." Bead-bound proteins were fractionated by SDS-PAGE under non-reducing conditions to avoid co-migration of the IgG heavy chain with NF-κB-p50. Reciprocal immunoblotting with an anti-NF-κB-p50 polyclonal antibody revealed that NF-κB-p50 was co-immunoprecipitated with PARP-1 (Fig. 6, lane 1). On the other hand, the PARP-1 antibody alone did not precipitate NF-κB-p50, as indicated by the absence of NF-κB-p50 signal in lane 2. It should also be mentioned that there was no cross-reactivity of the NF-κB-p50-specific antibody with PARP-1 (data not shown). Thus, Fig. 6 illustrates the innate ability of PARP-1 to form a protein complex with NF-κB-p50 in the absence of protein-poly(ADP-ribosyl)ation.

Due to the fact that all experimental data shown above was generated with a highly purified in vitro reconstituted system, we next proceeded to examine the validity of our observations in cultured HeLa cells following oxidative stress with hydrogen peroxide. We chose these genotoxic conditions, because hydrogen peroxide leads to the NF-κB activation (25) and because it can also activate the protein-poly(ADP-ribosyl)ation pathway, including automodification of PARP-1, via oxidative DNA damage conditions (27, 28).
control antibody (Pab421, 1/100, H9260 cells, albeit with less intensity (Fig. 7)). The results of this experiment are shown on Fig. 7. cells were treated with H$_2$O$_2$ to activate protein-

A lane 1

B lane 2

an antibody (e.g. anti-NF-

Effect of 3-AB on NF-κB DNA Binding in H$_2$O$_2$-treated HeLa Cells—HeLa cells were treated with H$_2$O$_2$ to activate protein-poly(ADP-ribose)ylation and NF-κB nuclear targeting. The results of this experiment are shown on Fig. 7. Fig. 7A shows that the treatment of HeLa cells with either 0.3 or 1.0 mM H$_2$O$_2$ (Fig. 7A, lanes 3 and 5) resulted in the enhanced formation of slowly migrating complexes with $^{32}$P-labeled NF-κB oligodeoxynucleotide. Arrows indicate positions of specific NF-κB DNA binding (NF-κB) and nonspecific DNA binding (n.s.). B, the specificity of DNA binding was confirmed by adding no antibody (lane 1), anti-NF-κB-p50 antibody (1 μg, lane 2), or control antibody (Pab421, 1 μg, lane 3) to the binding reaction and further incubation for 30 min at 4°C. After adding poly(dI-dC) and $^{32}$P-labeled NF-κB oligodeoxynucleotide and additional incubation for 15 min at room temperature, samples were electrophoresed through a 5% native polyacrylamide. Probe purity was confirmed by omitting nuclear extract from the incubation reaction mixture (lane 4).

The identity of the 3-AB-sensitive complex was determined to represent the NF-κB-DNA complex, because we observed its disappearance in the presence of a NF-κB-p50-specific polyclonal antibody (Fig. 7B, lane 2). Antibody cross-reactivity was excluded by the retention of the complex in the presence of control antibody (Fig. 7B, lane 3) and the complex originated from nuclear extract (Fig. 7B, lane 4). It is important to note that the inhibition of NF-κB binding by 3-AB is unlikely to be the result of extranuclear effects caused by this PARP-1-competitive inhibitor, because this compound failed to block the NF-κB activation in TNF-α-treated HeLa cells (data not shown). Therefore, data shown in Fig. 7 (A and B) confirm a directional relationship between protein-poly(ADP-ribose)ylation, e.g. PARP-1 automodification and NF-κB activation in hydrogen peroxide-treated HeLa cells as well.

**DISCUSSION**

In this study, we decided to assess the effect of covalent protein-poly(ADP-ribose)ylation on NF-κB DNA binding *in vitro*. To accomplish this goal, we co-incubated pure PARP-1 and the p50 subunit of NF-κB (NF-κB-p50) either in the presence or absence of βNAD$^+$. After the poly(ADP-ribose)ylation substrate was added to the mixture, the reaction was allowed to proceed, and NF-κB-p50 DNA binding was subsequently analyzed by EMSA. Our data consistently showed that NF-κB-p50 was able to bind to its oligodeoxynucleotide when PARP-1 was auto-poly(ADP-ribose)lated (Figs. 2–5). However, when PARP-1 was not poly(ADP-ribose)lated, NF-κB-p50 either formed a protein-protein complex with PARP-1 or the two proteins competed for DNA binding. Of these two possibilities, the former one was confirmed by our experiments carried out with H$_2$O$_2$-treated HeLa cells (Fig. 7, A and B). In these experiments, the DNA binding of endogenous dimeric NF-κB was facilitated under conditions where the automodification reaction of PARP-1, as well as other DNA binding proteins, was stimulated (Fig. 7). Furthermore, addition of 3-AB, a competitive inhibitor of protein-poly(ADP-ribose)ylation, also inhibited this effect despite the presence of a high concentration of endogenous HeLa cell nuclear DNA (Fig. 7B). Thus, our results are consistent with the conclusion that non-poly(ADP-ribose)lated PARP-1 forms a specific and stable protein complex with NF-κB. Interestingly, the co-immunoprecipitation of these two polypeptides in H$_2$O$_2$-treated HeLa cells was not possible, presumably because of the fact that most of the PARP-1 is proteolytically degraded by caspases during apoptotic execution (11) following oxidative DNA damage. Nevertheless, our *in vitro* co-immunoprecipitation experiments (Fig. 6), as well as those of others (32), strongly suggest that these two polypeptides physically interact with high specificity. In fact, co-immunoprecipitation experiments carried out *in vitro* with either βNAD$^+$ and/or 3-AB alone did not interfere with the PARP-1-NF-κB protein-protein interactions (data not shown).

We must note that other transcription factors such as YY1 and TBP/TFIIB apparently do not bind to their consensus oligodeoxynucleotide when covalently poly(ADP-ribosyl)ated (12) either. By contrast, in this study, NF-κB-p50 was not an efficient covalent target for poly(ADP-ribose)ylation (Fig. 4). In fact, in the presence of βNAD$^+$, NF-κB-p50 always bound to its oligodeoxynucleotide as a result of the auto-poly(ADP-ribose)ylation of PARP-1. It is possible that the protein poly(ADP-ribose)ylation-dependent inhibition of DNA binding observed with other transcription factors (12) might have been the result of electrostatic repulsion between the DNA probe(s) and poly(ADP-ribose) covalently bound to protein (38) as well.

At this point in time, we do not know which domain(s) of PARP-1 is(are) responsible for recognizing NF-κB. The modular structure of PARP-1 indicates that this polypeptide contains both a leucine zipper (LZ) and a BRCA1 C terminus motif (39, 42) in its automodification domain. Therefore, we speculate that PARP-1 may interact with other proteins, including NF-κB, via these peptide motifs. Indeed, it has been shown that numerous nuclear proteins, including histones, DNA polymerase α, tumor suppressor p53, DNA-dependent protein kinase, XRCC1 (x-ray repair cross-complementing 1), and oncogene product B-MYB are able to physically interact with PARP-1 *in vivo* and/or *in vitro* (9, 16, 19, 40–43). NF-κB-p50, on the other hand, is characterized by its Rel homology domain, through which the protein can dimerize with itself or with other Rel-family proteins (e.g. NF-κB-p65). NF-κB-p50 is also able to interact with different protein families, including basic LZ (bZip) proteins (e.g. NF-IL6 and C/EBP) and HMG proteins (44–46). However, whether PARP-1 interacts with NF-κB-p50...
via the Rel homology domain and/or LZ/BRCA1 C terminus motifs is not known. We think that the interaction of PARP-1 with NF-κB-p50 may result in either the masking of the DNA binding site or a conformational change of the latter with the resulting inhibition of its sequence-specific DNA binding properties. Further experiments to determine these two polypeptides recognize each other are in progress in our laboratory.

The regulation of NF-κB binding to its DNA by the auto- 
modication reaction of PARP-1 is not the only example that underscores a role for the highly efficient auto-poly(ADP-ribosyl)- 
ation of this enzyme in eucaryotic gene expression. In fact, as this manuscript was under preparation, Akiyama et al. (47) recently demonstrated that the activation of the Reg gene, a gene for insulin-producing β-cell regeneration, is also transcriptionally regulated by non-modiﬁed PARP-1, which appears to bind the NF-κB Reg promoter. Therefore, it is clear that the strength of the physical association of PARP-1 auto-poly(ADP-ribosylated PARP-1 is able to specifically bind NF-

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The Sequence-specific DNA Binding of NF-κB Is Reversibly Regulated by the Automodification Reaction of Poly (ADP-ribose) Polymerase 1

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