Transcriptional Coactivation of Nuclear Factor-κB-dependent Gene Expression by p300 Is Regulated by Poly(ADP)-ribose Polymerase-1*

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Nuclear factor κB (NF-κB) plays an important role in the transcriptional regulation of genes involved in inflammation and cell survival. In this study, we demonstrated that NF-κB-dependent gene expression was inhibited by E1A in poly(ADP)-ribose polymerase-1 knockout (PARP-1 (−/−)) cells complemented with wild type PARP-1 after tumor necrosis factor α (TNFα) or lipopolysaccharide (LPS) treatment. PARP-1 and p300 synergistically coactivated NF-κB-dependent gene expression in response to TNFα and LPS. Furthermore, PARP-1 interacted directly with p300 and enhanced the interaction of NF-κB1/p50 to p300. The C terminus, harboring the catalytic domain of PARP-1 but not its enzymatic activity, was required for complete transcriptional coactivation of NF-κB by p300 in response to TNFα and LPS. Together, these results indicate that PARP-1 acts synergistically with p300 and plays an essential regulatory role in NF-κB-dependent gene expression.

Nuclear factor κB (NF-κB) encompasses a family of inducible transcription factors including RelA/p65, RelB, c-Rel, p50, and p52 (reviewed in Refs. 1 and 2). These proteins share a conserved 300-amino acid region within their amino termini, designated the Rel-homology domain. This domain is responsible for dimerization, nuclear translocation, DNA binding and interaction with heterologous transcription factors. The specificity of NF-κB-dependent transcription is thought to be at least partially a result of differential homo- and heterodimerization of its family members, leading to a range of DNA-binding and activation potentials. The prototypical form, NF-κB, is a heterodimer consisting of the two subunits p50 and p65. The NF-κB family plays a key role in the transcriptional regulation of a variety of genes involved in inflammatory responses and cell survival. In addition, NF-κB has been associated with neurodegenerative processes and cancer (reviewed in Refs. 3 and 4). In most differentiated unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive transcription factor complex by its physical association with one of the several inhibitors of NF-κB (IκB). Treatment of cells with extracellular stimuli, such as cytokines, bacterial lipopolysaccharides (LPS), phorbol esters (phorbol 12-myristate 13-acetate), UV irradiation, γ irradiation, or potent oxidants leads to the rapid phosphorylation of IκB, which results in ubiquitination of IκB and subsequent degradation by the 26 S-proteasome pathway (5, reviewed in Ref. 6). Dissociation of NF-κB and IκB unmasks the nuclear localization sequences of the p65 and p50 subunits, allowing nuclear translocation, sequence-specific binding of NF-κB to κB sites, and activation of specific subsets of genes.

The number of transcriptional coactivator and cofactor families known to be involved in NF-κB-dependent gene expression is growing. The assembly of the NF-κB transcription complex is an important stage in NF-κB-dependent transcription and involves multiple coactivator/cofactor-NF-κB–DNA interactions (7–9). Key coactivators, the histone acetyltransferase p300 or its homologue, cAMP response element-binding protein binding protein (CBP) tightly associate directly with p65 (10, 11). These coactivators of NF-κB are essential for NF-κB-dependent gene expression (9, 12). p300 and CBP have also been shown to enhance transcriptional activation of various other transcription factors and to interact with TATA box-binding protein and general transcription factor IIB (13, reviewed in Ref. 14). Studies showed that protein kinase A-mediated phosphorylation of p65 is required for the recruitment of CBP by p65 (15).

Poly(ADP-ribose)polymerase-1 (PARP-1) is a nuclear homodimeric and chromatin-associated protein that catalyzes the transfer of ADP-ribose units from its substrate β-NAD⁺ to itself and other nuclear chromatin associated proteins (reviewed in Refs. 16 and 17). PARP-1 and NF-κB have both been suggested to play a crucial role in inflammatory disorders (18). Recent studies showed that PARP-1 knock-out (−/−) mice were protected against myocardial infarction, streptozotocin-induced diabetes, LPS-induced septic shock, zymosan-induced multiple organ failure, as well as collagen-induced arthritis, reaffirming the vital role of PARP-1 in inflammatory disorders (19–21, reviewed in Ref. 17). Growing experimental evidence suggests that PARP-1 can function as a transcriptional coactivator. PARP-1 has been shown to increase the transcriptional activity of TEF1/Max and AP-2 (22, 23). In addition, PARP-1 has been identified as an interaction partner of several transcription factors and cofactors, including TEF-1, Oct-1, AP-2, and PC3/topoisomerase-I (22–24). Meisterernst et al. have identified PARP-1 as an active component of the upstream factor stimulatory activity (USA)-derived positive cofactor PC1 (25). Earlier studies showed that the USA complex is essential for transcriptional activity of transcription factors such as NF-κB, SP1, and Oct-1 (26). Furthermore, several reports showed that the expression of NF-κB-dependent inflammatory genes such as TNFα, interleukin-1β, P-Selectin,
intercellular adhesion molecule, and inducible nitric-oxide synthase are impaired in PARP-1(-/-) mice after treatment with inflammatory stimuli as well as in mouse models of stroke and ischemia-reperfusion (reviewed in Ref. 17). Interestingly, Tulin and Spradling (27) recently found that Drosophila melanogaster mutants lacking normal PARP levels display immune defects similar to mice lacking the NF-κB subunit p50 (28). Furthermore, their results imply that the role of PARP-1 in NF-κB-dependent gene expression during immune responses has been conserved during evolution (27).

We recently provided evidence that PARP-1 can act as a coactivator for NF-κB in vivo (29, 30). We showed that PARP-1 was required for specific transcriptional activation of NF-κB in response to inflammatory stimuli. PARP-1 directly interacted through different domains with both subunits of NF-κB (p65 and p50) in vitro and formed a stable immunoprecipitable nuclear complex together with p50 and p65 in vivo. Remarkably, the enzymatic activity of PARP-1 was not required for full transcriptional activation of NF-κB in response to various stimuli. The exact molecular mechanisms of these observations are not yet clear.

In this study, we show that NF-κB-dependent gene expression in PARP-1(-/-) cells, transiently complemented with PARP-1, was inhibited by 12 S E1A. p300 alone was not sufficient for full transcriptional coactivation of NF-κB in these cells. The presence of PARP-1 was required for transcriptional coactivation of NF-κB in a stimuli-dependent manner. Furthermore, PARP-1 and p300 synergistically activated NF-κB in response to TNFα and LPS. In addition, PARP-1 formed a tight complex with p300 in vivo, and this interaction was direct in vitro. We showed that the previously described interaction of p65 with p300 was PARP-1-independent. However, the binding of PARP-1 to p300 provided an additional interaction site for p50 on p300. Further experiments revealed that the C terminus of PARP-1, but not its enzymatic activity, was required for full transcriptional coactivation.

Together, these results support the hypothesis that PARP-1 acts as an essential coactivator of NF-κB in concert with p300 to facilitate the formation and subsequent activation of the pre-initiation complex (PIC) in a stimuli-dependent manner.

MATERIALS AND METHODS

Plasmids—RSV-PARP-1, RSV-p300, RSV-12 S E1A wild-type, and RSV-12 S E1A-mutΔ32/36 expression vectors were described in Refs. 30 and 46. Expression vectors for GST-p300 fragments are described in Ref. 47. NF-κB-dependent luciferase reporter constructs pBII-luc and pB-luc, containing wild-type or mutated NF-κB binding sites, are described in Refs. 30 and 46. pHRSV-nt-β-gal is a descendant of Ref. 30. The PARP-1 deletion constructs pcDNA3.1-PARP-11-41, pcDNA3.1-PARP-141-531, and pcDNA3.1-PARP-1531-939 were obtained by digestion of the wild-type vector with EcoRI, EcoRV, and BamHI, respectively, and subsequent religation of the vector. Baculovirus for hPARP-1 was created with the BacPak System from BD Biosciences Clontech. Baculovirus hp300 was a generous gift from Dr. W. L. Kruus (Cornell University, Ithaca, NY).

Cell Culture, Transient Transfection, and Generation of Nuclear Extracts—Primary fibroblast cells isolated from lung of PARP-1(+/-) and PARP-1(-/-) mice, HeLa, and human embryonic kidney 293 cells were grown as described in Refs 13, 30, and 46. Cells were transfected as described previously except that primary cells were grown for 15 h in Dulbecco’s modified Eagle’s medium containing 3% fetal calf serum before stimulation with TNFα and LPS. The amount of DNA indicated in the figure legends was calculated for 10 ml of medium. Total amounts of DNA and equal molar ratios of promoters were kept constant in all set-ups by using empty vectors. For primary cells, only cell passages 3 to 6 were used for transfection experiments. Because of differences in transfection efficiencies, an expression plasmid of β-galactosidase (pHRSV-nt-β-gal) was co-transfected as a transfection efficiency control, and luciferase activities were normalized based on β-galactosidase activity. Luciferase activity was measured as described in Ref. 30. Nuclear extracts were prepared as described previously (11, 13, 46). Re-combinant human TNFα was obtained from R&D Systems, whereas LPS (0128 B12) was obtained from Sigma.

Immunoprecipitation and Immunoblotting—All immunoprecipitation and immunoblotting analysis for PARP-1, p65, p50, and p300 were performed as described previously (11, 30). Anti-RelA/p65 (C-30, sc-372), anti-p50 (H-119, sc-7178, C-19, sc-1190), anti-p300 (sc-2680), mouse IgG (sc-2025), rabbit IgG (sc-2027), and anti-c-MyC IgG (sc-2027) antibodies were obtained from Santa Cruz Biotechnology; anti-PARP-1 antibody (clone C2-10) was from Anaqua trading SA and anti-p300 antibody (14991A) was from BD PharMingen.

In Vitro Interaction and GST Pull-down Assays—Proteins fused to GST expressed in Escherichia coli were purified using glutathione-Sepharose beads according to the manufacturer’s protocols (Amersham Biosciences). All purified proteins were confirmed by Western blot analysis using the corresponding antibodies. Coupled in vitro transcription-translation reactions were carried out using the TNT T7-Quick system (Promega) according to the manufacturer’s protocol. GST pull-down assays were performed in the presence of 100 or 75 mM NaCl (low salt) or 150 mM NaCl (high salt) as described previously (30).

In Vitro Polynucleotide-virosisylation Assays—200 ng of baculovirus PARP-1 and 2 μg of baculovirus p300 were incubated with 200 ng of calf thymus DNA previously treated with DNase I in 100 μl containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 10 mM dithiothreitol, and 100 mM [γ-32P]NAD⁺ (100 nCi/ml). After 10 min of incubation at 30 °C, the reaction was stopped with hot 10% Laemmli buffer and analyzed on 8% SDS-PAGE. Gels were stained with Coomassie Blue, destained, dried, and autoradiographed on Kodak Bio-Max MS film.

RESULTS

PARP-1 Coactivation of NF-κB Is Repressed by 12 S E1A—Previous studies showed that p300/CBP is an important coactivator of NF-κB. 12 S E1A was shown to bind p300/CBP and thereby inhibit NF-κB-dependent transcription. To investigate the role of p300 in PARP-1(-/-) cells, we decided to examine whether 12 S E1A would inhibit the PARP-1 coactivator activity of NF-κB in vivo. We performed transient NF-κB-dependent reporter studies in primary fibroblast cells isolated from the lung of PARP-1(-/-) mice. Primary cells were co-transfected with the artificial NF-κB-dependent luciferase reporter constructs PBII- or pB-luc, containing wild-type or mutated NF-κB binding sites together with expression vectors for PARP-1 and 12 S E1A wild-type or 12 S E1A mutant and subsequently treated with the indicated stimuli (Fig. 1, A and B). Surprisingly, overexpression of wild-type 12 S E1A severely reduced NF-κB-dependent transcriptional activation by PARP-1 in primary PARP-1(-/-) cells in response to LPS and TNFα. However, overexpression of 12 S E1A mutant (12 S E1A-Δ32/36), which is unable to bind p300/CBP, did not inhibit NF-κB-dependent transcriptional activation by PARP-1. Because PARP-1 did not interact physically with 12 S E1A (data not shown), these experiments suggested that p300/CBP is likely to be functionally linked to PARP-1.

PARP-1 Is Required for Transcriptional Coactivation of NF-κB by p300 in Response to LPS and TNFα—To further investigate the relevance of p300 on PARP-1 coactivated NF-κB-dependent gene expression, primary fibroblast cells isolated from the lung of PARP-1(+/-) or PARP-1(-/-) mice were transfected with the indicated NF-κB-dependent luciferase reporter constructs together with expression vectors for p300 and/or PARP-1 and subsequently treated with the indicated stimuli (Fig. 2). The experiments revealed that overexpression of p300 and PARP-1 had no effect on basal NF-κB-dependent transcriptional activation in both primary PARP-1(+/-) and PARP-1(-/-) cells (Fig. 2A). Although overexpression of p300 alone augmented NF-κB-dependent transcriptional activation in primary PARP-1(+/-) cells—3-fold in response to LPS or TNFα (Fig. 2, B and C, left), no NF-κB-dependent transcriptional activation by p300 was observed in primary PARP-1(-/-) cells after LPS or TNFα treatment (Fig. 2, B and C, right). However, additional co-expression of p300 with PARP-1
PARP-1 Interacts with p300

PARP-1 Interacts with p300—As these results strongly suggested that p300 and PARP-1 might physically interact in vivo, we immunoprecipitated PARP-1 complexes from nuclear extracts using antibodies directed against PARP-1 and tested the presence of p300 and PARP-1 by immunoblot analysis using an anti-PARP-1 or anti-p300 antibody. p300 indeed formed a tight complex with PARP-1 (Fig. 3A, left). DNA did not mediate the association of p300 and PARP-1, because the presence of ethidium bromide or DNase1 did not affect p300/PARP-1 binding (data not shown). Reciprocal immunoprecipitation experiments confirmed the previous observation (Fig. 3A, right).

We proceeded to map the interaction domains of p300 and PARP-1, respectively. GST-fusion protein pull-down experiments were performed using different p300 fragments and nuclear extracts from primary PARP-1 (+/−) cells, which were untreated or treated with TNFα. Subsequent immunoblot analysis using an anti-PARP-1 antibody revealed that PARP-1 was binding to p300 fragments 1 and 4 under unstimulated conditions (Fig. 3B, top left) and also after TNFα treatment to p300 fragment 5 (Fig. 3B, bottom left). These p300 fragments correspond to amino acid residues 1-625, 1639-1891, and 1830-2414 of p300, respectively.

To map the p300-interaction domain of PARP-1, we in vitro-translated truncated forms of PARP-1 and tested their ability to interact with different GST-p300 fragments (Fig. 3C, Input). The experiments revealed that only GST fragments 1 and 4 of p300 interacted with PARP-1 under stringent conditions (see “Materials and Methods”). Under non-stringent conditions, PARP-1 also bound to p300 fragment 5, although no interaction with p300 fragments 2 and 3 could be observed (Fig. 3C, data not shown). The minimal region of interaction with p300 fragment 1 was limited to the N-terminal 341 amino acids of PARP-1. In contrast, the interaction with p300 fragments 4 and 5 seems to be limited to aa 531–829 of PARP-1. These results suggest that two distinct interactions are required for full interaction of PARP-1 and p300: an N-terminal interaction that involves aa 1–625 of p300 and aa 1–341 of PARP-1 as well as a C-terminal interaction that requires aa 1639–2414 of p300 and aa 531–829 of PARP-1.

To determine which domain of PARP-1 was important for NF-κB coactivator function in response to inflammatory stimuli, we performed cotransfection experiments in primary PARP-1(−/−) cells using a NF-κB-dependent luciferase reporter construct and different deletion forms of PARP-1 (Fig. 3D). Although transfection of wild-type PARP-1 fully restored TNFα-induced NF-κB-dependent transcriptional activation, cotransfection of different deletion mutants of PARP-1 revealed that expression of a deletion mutant of PARP-1 (expressing aa 1–531) was not able to stimulate NF-κB-dependent gene expression at all (Fig. 3D). Identical experiments with subsequent LPS treatment revealed the same results (data not shown). The inability of this deletion mutant to stimulate NF-κB-dependent transcriptional activation might be explained by its inability to interact with the C terminus of p300, implying the importance of this second interaction site at the C terminus of p300 (Fig. 3, compare C and D). The other deletion mutant of PARP-1 (expressing aa 1–829) was able to stimulate NF-κB-dependent gene expression but not to the same extent as the

![Figure 1](http://www.jbc.org/Downloadedfrom)
FIG. 2. PARP-1 synergistically activates together with p300 NF-κB-dependent gene expression in a stimuli-dependent manner. Primary PARP-1(+/-) or PARP-1(-/-) fibroblasts were cotransfected with pBII-luc or pf-luc (1 µg) and RSV-nt-β-Gal (200 ng) together with RSV-p300 (2 µg), CMV-P/CAF (1 µg), RSV-PARP-1 (2 µg), or RSV empty vector, as indicated. Cells were either untreated (A) or stimulated 24 h after transfection with LPS (10 µg/ml) (B) or TNFα (10 ng/ml) (C and D) for 8 h. Cells were harvested 32 h after transfection and NF-κB-dependent gene expression determined as described under “Materials and Methods.” The indicated activation was determined as described in Fig. 1A. Error bars indicate S.E. of three independent experiments.
full-length PARP-1 (Fig. 3D). Given that mutant aa 1–829 was able to bind to the N and C termini of p300, these results indicate that the interaction of PARP-1 with p300 is important for transcriptional activation of NF-κB but that the C-terminal part of the catalytic domain of PARP-1 seems to play an additional important role for the transcriptional coactivation by PARP-1 that is independent of p300. However, we cannot exclude the possibility that the observed decrease in coactivation

Fig. 3. PARP-1 interacts with p300 in vitro and in vivo. A, PARP-1 and p300 form a complex in vivo. PARP-1 and p300 were coimmunoprecipitated (IP) in the presence of 120 mM NaCl from nuclear extract of TNFα-treated (30 min) HeLa cells using control IgGs, an anti-PARP-1 (left), and anti-p300 IgG (right). Bound proteins were resolved by SDS-PAGE and subsequently detected by immunoblot (IB) analysis for PARP-1 and p300. Input lanes represent 1% of the input. B, PARP-1 binds two different domains of p300. Pull-down assays with the indicated p300 fragment fused to GST (0.8 μg) and nuclear extract from untreated (top right) or TNFα-treated HeLa cells (center right). Bound proteins were resolved by SDS-PAGE followed by subsequent immunoblot analysis for PARP-1. Input lanes represent 1% of the input. The Coomassie gel of the expressed domains of p300 is shown at bottom right. A schematic representation of the p300 fragments is shown at left. C, mapping of p300 interaction domain on PARP-1. GST (0.8 μg) or GST-p300 fragments (0.8 μg) were incubated with in vitro-translated PARP-1 full-length (FL) or different deletion mutants (as indicated) in the presence of 150 mM NaCl for fragments p300–1 and p300–4 or 100 mM NaCl for fragment p300–5. Bound complexes were resolved by SDS-PAGE and visualized by autoradiography. Input lanes represent 2.5% of the input. D, aa 531–1014 of PARP-1 are essential for NF-κB-dependent gene activation. Cells were cotransfected with pBII-luc (black bars) (1 μg) or pf-luc (white bars) (1 μg) and RSV-nt-β-Gal (200 ng) together with PARP-1 wild-type (2 μg) or deletion constructs aa 1–341 (0.5 μg), aa 1–531 (0.8 μg), or aa 1–829 (1.5 μg) and subsequently treated for 8 h with TNFα (10 ng/ml) (left). The indicated activation was determined as described in Fig. 1A. Error bars indicate S.E. of three independent experiments. The expression of PARP-1 full-length (WT) and different deletion mutants of PARP-1 in PARP-1 (−/−) cells is shown in the right. Different amounts of DNA were included to work with a comparable molarity of DNA per construct and experiment. PARP-1 (−/−) cells were transfected with PARP-1 wild type (10 μg) or deletion constructs aa 1–341 (2.5 μg), aa 1–531 (4 μg), or aa 1–829 (7.5 μg). 48 h after transfection, cells were harvested, and nuclear extracts were analyzed by SDS-PAGE and subsequent immunoblot analysis for the different PARP-1 forms.
by PARP-1 mutant aa 1–829 might be caused by a difference in conformation or stability of the PARP-1 deletion mutant.

PARP-1 Mediates the Interaction between NF-κB/p50 and the C-terminal Domain of p300—The association of PARP-1 with p300 and NF-κB through two different domains raised the question of whether the observed synergistic activation of NF-κB by PARP-1 and p300 was caused by PARP-1 influencing the ability of NF-κB (p65/p50) to associate with p300. To determine whether PARP-1 affected NF-κB binding to p300, p300 was immunoprecipitated from nuclear extracts of TNFα-treated PARP-1(+/-) and (-/-) cells and tested for the presence of p65 and p50 by Western blot analysis using the indicated antibodies (Fig. 4A). In PARP-1(+/-) extracts, p300 was associated with PARP-1 and both subunits of NF-κB (Fig. 4A, left). Immunoblot analysis of immunoprecipitates from PARP-1(+/-) extracts revealed that despite the absence of PARP-1, p65 and p50 still formed a complex with p300 (Fig. 4A, right). However, p50 bound to p300 to a much lesser extent when immunoprecipitated from PARP-1(-/-) extract compared with PARP-1(+/-) extracts (Fig. 4A, bottom). Together, these experiments revealed that NF-κB could still form a complex with p300 after induction by TNFα, although the relative amount of the two bound NF-κB subunits (p65 and p50) was reduced in PARP-1(-/-) cells. Importantly, because under the same conditions no transcriptional activation was observed in the previous experiments (Figs. 1 and 2), these experiments suggested that the binding alone of NF-κB to p300 was not sufficient for NF-κB-dependent gene expression in response to TNFα and LPS or that small differences in NF-κB binding to p300 led to large differences in NF-κB-dependent transcription.

To further investigate whether PARP-1 would influence NF-κB binding to p300 we performed GST-fusion protein pull-down experiments using different p300 fragments and extracts from PARP-1(+/-) and PARP-1(-/-) cells untreated or treated with TNFα. p65 bound to the p300 fragment 1 in unstimulated cells and to p300 fragments 1, 4, and 5 in TNFα-stimulated cells, although to a much lesser extent to p300 fragment 4 (Fig. 4B). The observed binding of p65 (and also p50) to p300 fragment 1 in unstimulated cells might represent the basal NF-κB activity in these cells. As previously observed, all interactions of the p65 subunit to the p300 fragments were not dependent on PARP-1 (Fig. 4B, compare left and right). p50, (like p65) bound in unstimulated cells to p300 fragment 1 in a PARP-1-independent manner. However, after TNFα stimulation, p50 interacted with p300 fragments 1, 4, and 5 in the presence of PARP-1, but failed to bind to p300 fragment 5 when PARP-1 was absent (Fig. 4B, compare bottom left and bottom right). This observation suggested that PARP-1 might stabilize the binding of p50 to p300 fragment 5 after TNFα stimulation.

To analyze the ability of both NF-κB subunits to bind separately to p300, repeated GST-fusion protein pull-down assays with p300 fragments and in vitro transcribed/translated p50 and p65 were performed. p65 interacted with p300 fragments 1, 4, and 5 (Fig. 4C, top). Identical experiments under stringent conditions revealed that binding of p65 to p300 fragment 1 was much stronger than to p300 fragments 4 and 5 (data not shown). These findings are consistent with previous observations, which described RelA/p65 as directly interacting with the N terminus (C/H1 domain) as well as the C-terminal region of CBP (10). In vitro transcribed/translated p50, on the other hand, interacted with p300 fragment 1 and to a weaker extent with p300 fragment 4 but not with p300 fragments 2, 3, or 4 (Fig. 4C, bottom). This interaction pattern was similar to the one observed with nuclear extracts from PARP-1(+/-) cells (Fig. 4D, bottom). Together, these results suggested that p50 and p65 interacting directly and independent of each other with distinct domains of p300 1, 4 and 5 or 1 and 4, respectively.

To determine whether PARP-1 could influence the binding of p50 to fragment 5 (as observed in Fig. 4B), we repeated the GST-fusion protein pull-down experiments with p50 in the absence or presence of in vitro transcribed/translated PARP-1 (Fig. 4D). PARP-1 and p50 bound independently of each other to p300 fragment 4. Pull-down experiments with p300 frag-
ment 5, however, revealed that the binding of p50 was strictly dependent on the presence of PARP-1 (Fig. 4D), confirming the observations that p50 would interact more weakly or not at all with the C terminus of p300 in the absence of PARP-1 (Fig. 4, A and B). Interestingly, PARP-1 was able to interact with p300 fragment 5 alone, suggesting that the binding to p300 fragment 5 after TNFα treatment (observed in Fig. 3B) was regulated by a stimuli induced modification of PARP-1 rather than by another protein, such as p65. Together, these results confirmed that the complex formation of PARP-1 with p300 generated an additional site of interaction for NF-κB with p300.

**p300 Is Not Poly(ADP-ribosylated) and the Enzymatic Activity of PARP-1 Is Not Required for Full Transcriptional Coactivation of NF-κB by p300**

We have previously shown that the enzymatic activity of PARP-1 was not required for full transcriptional activation of NF-κB by p300 (17). To re-evaluate the functional relevance of the enzymatic activity of PARP-1 in the presence of p300 and to confirm our previous results, we investigated whether p300 might be poly(ADP)-ribosylated by PARP-1. In vitro Poly(ADP)-ribosylation assays were performed using recombinant PARP-1 and recombinant p300 full-length, purified from insect cells, in the presence of [32P]-NAD+ (Fig. 5A). The experiments revealed that p300 was not poly(ADP)-ribosylated by PARP-1 in vitro, at least under the tested conditions, although PARP-1 automodification was easily detected.

To further investigate the importance of PARP-1’s enzymatic activity in p300-mediated transcriptional coactivation of NF-κB, transient NF-κB-dependent reporter studies in primary PARP-1(-/-) fibroblasts were performed (Fig. 5, B and C). Primary PARP-1(-/-) cells were cotransfected with pBII-Luc (1 μg) (black bars) or pf-luc (1 μg) (white bars) and RSV-nt-β-Gal (200 ng) together with expression constructs for p300 (2 μg) and PARP-1 wild type (2 μg) or enzymatic mutant E988K (2 μg). 32 h after transfection, cells were subsequently treated or untreated for 8 h with TNFα (10 ng/ml) (B) or LPS (10 μg/ml) (C). The indicated activation was determined as described in Fig. 1A. Error bars indicate S.E. of three independent experiments.

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**Fig. 5. Enzymatic activity of PARP-1 is not required for full transcriptional coactivation of NF-κB by p300.** A, p300 is not poly(ADP)-ribosylated by PARP-1 in vitro. *In vitro* Poly(ADP)-ribosylation assays were performed with 200 ng of recombinant PARP-1 and 2 μg of recombinant p300 full-length, purified from insect cells, using [32P]-NAD+ in the presence of 100 mM NaCl as described under “Materials and Method.” Coomassie gel is shown at right. B and C, enzymatic activity of PARP-1 is not required for full transcriptional coactivation of NF-κB by p300. Primary PARP-1(-/-) cells were co-transfected with pBII-luc (1 μg) (black bars) or pf-luc (1 μg) (white bars) and RSV-nt-β-Gal (200 ng) together with expression constructs for p300 (2 μg) and PARP-1 wild type (2 μg) or enzymatic mutant E988K (2 μg). 32 h after transfection, cells were subsequently treated or untreated for 8 h with TNFα (10 ng/ml) (B) or LPS (10 μg/ml) (C). The indicated activation was determined as described in Fig. 1A. Error bars indicate S.E. of three independent experiments. mut, mutant; WT, wild type.
PARP-1 interacts with p300

PARP-1 interacts with p300 and PARP-1 mediates an additional interaction site of PARP-1, but not its enzymatic activity, is required for interaction with p300 for complete transcriptional coactivation of NF-κB.

In previous reports, we have shown that NF-κB-dependent gene activation is impaired in primary PARP-1(−/−) cells and that PARP-1 acts as a coactivator of NF-κB in vivo, independent of its enzymatic activity (29, 30). The main findings of this study are the physical and functional association of PARP-1 with p300. These studies provide evidence that recruitment of PARP-1 in addition to p300 is required for NF-κB-dependent transcription in response to a subset of inflammatory stimuli. More intriguingly, we have found that the C-terminal domain of PARP-1, but not its enzymatic activity, is required for interaction with p300 for complete transcriptional coactivation of NF-κB.

Virtually all cell types show NF-κB responses in which the activity of NF-κB is regulated at multiple levels: synthesis, subcellular localization, posttranslational modification, differential dimerization, DNA binding, and interaction with a specific combination of coactivators (reviewed in Refs. 31 and 32). Unsurprisingly, the genes activated by NF-κB also vary depending on the coactivator sets present in the cell. Previous reports have shown that NF-κB-dependent transcriptional complexes seem to require not only the coactivator p300 but its structural homologue CBP but also the P/CAF and the p160 family of steroid receptor coactivators (9, 33, 34). It is thought that the critical role of p300/CBP and its associated coactivators is to promote the rapid formation of the pre-initiation and re-initiation complexes which facilitate multiple rounds of transcription by modifying the amino-terminal tails of nucleosomal histones and bridging NF-κB to the general transcriptional machinery.

However, although the recruitment of p300 or CBP to NF-κB-dependent enhancosomes is required for synergistic activation, tethering p300/CBP alone to the promoter through NF-κB might not be sufficient for full activity of NF-κB in the context of chromatin. Several reports indicated that the combined actions and interactions of distinct transcriptional coactivator complexes and cofactors seem to be attributable to the strong transcriptional activity of NF-κB, depending on the stimuli and the cell type (9, 26, 35–37). Because other sequence-specific activators also use p300/CBP and their associated coactivators, the expression levels and localization of these coactivators will vary between different cell types and could be limiting in vivo. Additional components might be required to stabilize the association of distinct NF-κB coactivator complexes.

Indeed, an impaired NF-κB-dependent transcriptional gene activation could be detected in PARP-1(−/−) cells in response to TNFα and LPS compared with wild-type cells, despite the fact that p300 and p65/RelA would form a comparable complex in these cells. Thus, the interaction of PARP-1 with p300 seems to be important for NF-κB-dependent transcriptional activation in response to pro-inflammatory stimuli. The regions of PARP-1 that are responsible for the association with the N- and C-terminal domains of p300 indicated that the recognition of PARP-1 by p300 involves two domains of PARP-1 that are completely distinct from each other. Stimulation of cells with TNFα induced an additional PARP-1 binding site in the C-terminal domain of p300. Whether the stimuli-dependent interaction of PARP-1 with the very C-terminal part of p300 might be caused by modification of PARP-1 in vivo has to be investigated. Surprisingly, the C-terminal interaction between p300 and PARP-1 mediates an additional interaction site of p300 with p50, the small subunit of NF-κB in response to TNFα. The importance of this C-terminal interaction was confirmed by the complementation experiments with different PARP-1 deletion mutants. The establishment of a stable interaction between the NF-κB subunits p65 and p50 with p300 seems to be a crucial prerequisite for efficient transcription by NF-κB in response to TNFα and LPS. The use of a bivalent interaction between NF-κB1 subunits and p300 apparently indicates a more stringent control in the recruitment of p300 to nuclear NF-κB complexes. However, the physiological relevance of this additional interaction of p300 and p50 has yet to be investigated in vivo.

Interestingly, we could show that p300, as previously shown for NF-κB, was not poly(ADP)-ribosylated by PARP-1 in vitro under the conditions tested. In addition, the enzymatic activity of PARP-1 was not required for full transcriptional activation of NF-κB in the presence of p300. Our results are also in agreement with previous observations that the C-terminal domain of PARP-1, harboring the catalytic domain, is important for transcriptional regulation (27). The observation that the enzymatic inactive PARP-1 mutant was able to complement PARP-1(−/−) to the same extent as wild-type PARP-1 provides strong evidence that the protein structure of the catalytic domain of PARP-1 is more important than its catalytic activity for transcriptional coactivity. However, we cannot yet exclude the idea that the enzymatic activity of PARP-1 might be critical for the modulation of the chromatin structure for the activation of NF-κB-dependent genes in different chromatin environments in vivo (38).

Earlier studies showed that full activation of transcription by NF-κB in cell-free systems required a crude precursor USA coactivator fraction in addition to general transcription factors (26). The crude precursor human USA fraction consists of at least six independent subfractions of positive cofactors: PC1/PARP-1, PC2/Mediator-like complex; PC3/topoisomerase-I; PC4/ssDNA binding protein; PC52; PC6; and HMG2 (25, 39–41, reviewed in Refs. 42 and 43). PC1/PARP-1 was thought to provide, together with the other USA-derived positive cofactors PC3, PC4, PC52, and HMG2, a structural/architectural function in assembling and stabilizing the PIC (25). Our previous report that the enzymatic activity of PARP-1 was not required for full transcriptional activation of NF-κB in response to various stimuli supports this hypothesis. Moreover, because PC1/PARP-1, PC3, and PC4 are all non–sequence-specific DNA binding proteins, it was suggested that these cofactors function by affecting the accessibility of RNA polymerase II to chromatin (43).

Our studies suggest that the assembly of the PIC in primary PARP-1(−/−) cells might be defective in response to inflammatory stimuli. Further analysis revealed that PARP-1 did not physically interact with TFIIIB, TATA box-binding protein, and various TATA box-binding protein-associated factors (data not shown). These results are consistent with the observation of Meisterernst et al. (25) that in vitro transcription assays, PARP-1 is only required during assembly of RNA polymerase II and general transcription factors on preformed TFIIID-TFIIA-DNA complexes. PARP-1 was unable to stimulate the formation of a TFIIID-promoter complex. In addition, in their studies, PARP-1 stimulated transcription only when added before the complete PIC was formed (25), which implies that PARP-1 might only function during assembly of the PIC. PARP-1 together with other structural/architectural positive cofactors could facilitate the co-operative interactions between sequence-specific activators and different coactivator complexes, thereby providing an architectural function in stabilizing the pre-initiation complex. The fact that PARP-1(−/−) mice do not show the same phenotype as RelA/p65(−/−) animals (44, 45) indicates that only a subset of NF-κB target genes are dependent on PARP-1. Thus, in other contexts, the requirement for PARP-1
will be bypassed through other specific coactivators (36, 37) and structural/architectural cofactors, most probably in a cell type and stimuli-dependent manner.

Taken together we propose that PARP-1 is an essential structural/architectural positive cofactor of NF-κB and that PARP-1 acts in concert with p300 to help facilitating the formation and subsequent activation of the pre-initiation complex (PIC) in a stimuli-dependent manner. These results will also give a new insight into the function of PARP-1 and NF-κB during pathological processes in inflammatory disorders such as streptozotocin-induced diabetes and lipopolysaccharide (LPS)-induced septic shock as observed with PARP-1(+/−) mice (17).

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