Evidence for regulation of NF-κB by poly(ADP-ribose) polymerase

Masanori KAMEOKA*, Katsuya OTA*, Toshifumi TETSUKA†, Yasuharu TANAKA†, Asako ITAYA*, Takashi OKAMOTO† and Koichiro YOSHIHARA*[1]

*Department of Biochemistry, Nara Medical University, Kashihara, Nara 634-8521, Japan, and †Department of Molecular Genetics, Nagoya City University Medical School, Kawasumi, Nagoya 467-8601, Japan

The DNA-binding activity of NF-κB in nuclear extracts of poly(ADP-ribose) polymerase (PARP)-defective mutant L1210 cell clones was markedly increased and was inversely correlated with the PARP content in these cells. The DNA-binding activity of NF-κB in a clone with the lowest PARP content (Cl-3527, contained 6% of PARP of wild type cells) was about 35-fold of that of the wild-type cells, whereas the change in the DNA-binding activity of AP-1 and SP-1 in the mutant was relatively small or not so significant. Transfection of a PARP-expressing plasmid to the mutant cells decreased the abnormally high levels of NF-κB complexes, especially p50/p65(Rel A) complex, to near the normal level. Moreover, poly(ADP-ribosyl)ation of nuclear extracts *in vitro* suppressed the ability of NF-κB to form a complex with its specific DNA probe by approx. 80%. Further analysis with purified recombinant NF-κB proteins revealed that both rp50 and rMBP-p65 (Rel A) proteins, but not rGST-IκB, could be poly(ADP-ribosyl)ated *in vitro* and that the modification resulted in a marked decrease in the DNA-binding activity of rMBP-p65, whereas a slight activation was observed in rp50. Poly(ADP-ribosyl)ated p65/NF-κB was detected in the cytosol of wild type L1210 cells by immunoblotting with anti-poly(ADP-ribose) and anti-p65 antibodies. Taken together, these results strongly suggest that PARP is involved in the regulation of NF-κB through the protein modification.

Key words: p65, p50, transcription factor, L1210.

INTRODUCTION

Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme, that catalyses a successive transfer of ADP-ribose moiety of NAD^+ to a protein, thus forming a protein, bound polymer of ADP-ribose [1]. Various nuclear proteins including histones [1], HMG proteins [2] and nuclear enzymes involved in DNA metabolism, such as Ca^{2+}, Mg^{2+}-dependent endonuclease [3,4], DNA topoisomerases [5,6], DNA ligase I [7,8], terminal deoxynucleotidyl transferase [9], DNA polymerases [8,10] and PARP itself (automodification; [11,12]) have been shown to be the acceptors for poly(ADP-ribose).

Several lines of evidence suggest that PARP is involved in various cellular functions including DNA repair [13,14], DNA replication [15,16], genetic recombination [17], and cell death [18–20] (for review see [21–23]). Recent studies with mice lacking PARP-gene also indicated that PARP is required for the recovery of cells from DNA damage [24] and is important for keeping genomic stability [25]. It is also shown that PARP is essential for the progression of some types of cell death including neuronal cell death following cerebral ischaemia [26] and pancreatic β cell death by streptozotocin [27,28]. Although it is reported that mice lacking PARP-gene showed almost normal development [24,29], the finding should be re-evaluated since Amé et al. [30] recently reported that the PARP (PARP-1)-gene disrupted mice expressed 10–15% of residual PARP activity owing to the expression of the second PARP gene (PARP-2).

Several recent studies suggested that PARP is involved more directly in the regulation of the transcription of some genes [31–34]. In order to study the relation between PARP and transcription factors, we examined whether a PARP-defective mutant cell clone, which had been established in our laboratory [35], was expressing normal levels of transcription factors or not, and found that the mutant expressed extremely high levels of activated NF-κB, a transcription factor involved in the regulation of a large number of genes of cells and viruses [36]. Further analyses revealed that PARP and active NF-κB levels in various PARP-defective L1210 cell clones were inversely correlated. Thus, we examined more precisely the effect of poly(ADP-ribosyl)ation on the activity of NF-κB with the use of purified poly(ADP-ribosyl)ating enzyme system and recombinant NF-κB proteins. Several lines of evidence suggesting that PARP is involved in the regulation of NF-κB are presented in the present report.

EXPERIMENTAL PROCEDURES

Materials

Labelled compounds

[*Adenine*-2,8-^{3}H]NAD^+ was purchased from Moravek Biochemicals Inc. (Brea, CA, U.S.A.); [*Adenylate*-^{32}P]NAD^+ (^{32}P-NAD^+) and [γ-^{32}P]ATP were purchased from NEN Life Science Products Inc. (Boston, MA, U.S.A.).

Antibodies

Anti-NF-κB p50 (C-19) (anti-p50) and anti-NF-κB p65 (SC-109) (anti-p65) polyclonal antibodies were purchased from Santa

Abbreviations used: 3AB, 3-aminobenzamide; EMSA, electrophoretic mobility shift assay; PARP, poly(ADP-ribose) polymerase; pADPR, poly(ADP-ribose); PMA, phorbol 12-myristate 13-acetate; MBP, maltose binding protein; IPTG, isopropyl-1-thio-beta-D-galactopyranoside; GST, glutathione S-transferase; TMDT and TDT, reaction mixtures for PARP with and without 10 mM $MgCl_2$ respectively; DTT, dithiothreitol.

[1] To whom correspondence should be addressed: (e-mail: kyoshiha@naramed-u.ac.jp).

Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antiserum against poly(ADP-ribose) (anti-pADPR) was prepared in our laboratory by immunizing a rabbit with a mixture of purified poly (ADP-ribose) and methylated bovine serum albumin (1.32 mg each) in 1:1 emulsion with Freund's complete adjuvant. Anti-PARP antibody was prepared as described previously [35].

Recombinant proteins

Recombinant human NF-κB p50 (rp50) was purchased from Promega (Madison, WI, U.S.A.). Other recombinant proteins, human rMBP-p65/Rel A (rMBP-p65), human recombinant IκB-α (rGST-IκB), and a recombinant antioncogene product of rMBP-p53 were prepared as follows. rMBP-p65 and rMBP-p53, expressed as fusion proteins with N-terminal maltose binding protein (MBP), were produced in *E. coli* DH5α, and purified using an affinity chromatography. Briefly, cDNAs for p65 and p53 were subcloned into the MBP expression vector, pMAL-c2 plasmid (New England Biolabs, Beverly, MA, U.S.A.), and transformed to *E. coli*. MBP-fusion proteins were induced with 0.4 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) at 30 °C for 3 h. Then, MBP fusion proteins were purified with amylose resin, as suggested in manufacturer's protocol. pMAL-c2 expressing MBP-LacZ (rMBP-LacZ) was from New England Biolabs. rMBP-LacZ was expressed and purified as in the case of rMBP-p65. Recombinant IκB α (rGST-IκB) was expressed as a fusion protein with N-terminal glutathione S-transferase (GST). Briefly, cDNA for IκB α was subcloned into expression vector, pGEX-4T-3 (Pharmacia–Amersham), and transformed to *E. coli* DH5α. GST-fusion protein was induced with 0.4 mM IPTG at 30 °C for 3 h. Then, the GST fusion protein was purified with glutathione-Sepharose 4B as indicated in manufacturer's protocol. The cDNA for wild-type p53 was a generous gift from Dr. B. Vogelstein (Johns Hopkins Oncology Center, U.S.A.). The human p65 (rel A) and the human IκB α (MAD-3) cDNAs were obtained from human lymphnode cDNA library using PCR.

Other materials

Poly(dI-dC) was purchased from Pharmacia-Amersham (Uppsala, Sweden). 3-Aminobenzamide (3-AB) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine thymus PARP [37], activated DNA [38], and pADPR [39] were prepared as described, respectively.

Cells and cell culture

Murine lymphocytic leukaemia cells, L1210 and L1210-derived PARP deficient cell clones, Cl-3, Cl-352, Cl-3527 and Cl-3527R [35] were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in a CO_2-incubator at 33 °C.

Electrophoretic mobility shift assay (EMSA)

The double-stranded oligonucleotides with the sequences of the binding sites for NF-κB, SP-1 and AP-1 were purchased from Promega. These oligonucleotides were end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Takara Shuzo Co. Ltd., Kyoto, Japan) and used as the probes. Preparation of nuclear extracts was carried out, according to the previous report [40]. Nuclear extracts (2–10 μg protein) were pre-incubated in a total volume of 24 μl of a reaction mixture (EMSA mixture) containing 20 mM Hepes/KOH, pH 7.8, 168 mM KCl, 1 mM EDTA, 1 mM/dithiothreitol (DTT), 2 mM $MgCl_2$, 8% glycerol, 0.2 mM PMSF, and 2 μg of poly(dI-dC) for 15 min on ice and then the sample was mixed with 1 μl of ^{32}P-labelled probe (0.5 ng, approx. 50000 cpm) and incubated for additional 30 min at room temperature. Competition assays were performed as described previously [40]. For supershift analysis with antibodies, the nuclear extracts were pre-incubated as above except that the EMSA mixture contained either anti-p50, anti-p65 or control IgG in addition to all of the components as above. The EMSA mixture for purified recombinant proteins, rp50 or rMBP-p65 proteins, were slightly modified as follows: A 20 μl-reaction mixture contained 10 mM Hepes/KOH, pH 7.8, 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol, 0.05% NP 40, 0.2 mM PMSF, 50 ng of the recombinant protein tested, and 25 ng of ^{32}P-labelled probe (specific radioactivity: 50000 cpm/25 ng). After the reaction, the DNA–protein complexes were separated on 6% (for NF-κB) or 4% (for AP-1 and SP-1) polyacrylamide gels in the cold room, then the gels were dried and the radioactive bands were visualized with X-ray film.

Poly(ADP-ribosyl)ation

The nuclear extracts or purified recombinant NF-κB proteins were incubated in a purified poly(ADP-ribosyl)ating enzyme system containing the indicated concentrations of purified PARP, NAD^+ and activated DNA, 25 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT and 0.05% Triton X100 (TMDT). In some experiments, either $MgCl_2$ was omitted from the reaction mixture (TDT) to limit the chain elongation of pADPR [41] or unlabelled NAD^+ was replaced by [^{32}P]NAD^+, as indicated in the respective experiment. The reaction was carried out at 25 °C for 30 min and terminated by the addition of a final concentration of 2.5 mM 3-AB.

Immunoblot

The transblot of samples on a gel to PVDF membrane was carried out as described previously [42]. After blocking with 2.5% non-fat milk in PBS, the blots were immuno-stained by successive treatment with an appropriately diluted primary antibody and peroxidase-labelled secondary antibody, and then the immuno-complex was located using ECL Western blotting detection reagents (Pharmacia–Amersham).

Densitometry

A relative value of the amount of a shift-band of EMSA, which was visualized on the X-ray film, was determined by densitometry using NIH Image 1.61 software.

RESULTS

Active NF-κ-B level is dramatically increased in the nuclear extracts of PARP-defective L1210 cell clones

Several recent reports [32–34,43,44] have suggested that PARP is directly involved in the regulation of some genes through the interaction of this enzyme with various transcription factors. To approach this subject, we examined NF-κB in the PARP-defective, L1210-derived cell clones which had been established in our laboratory [35].

It is well known that NF-κB-related protein family is located in cytosol as an inactive complex such as p50/p65(Rel A)/IκB [36] and the complex is activated by dissociation of IκB through

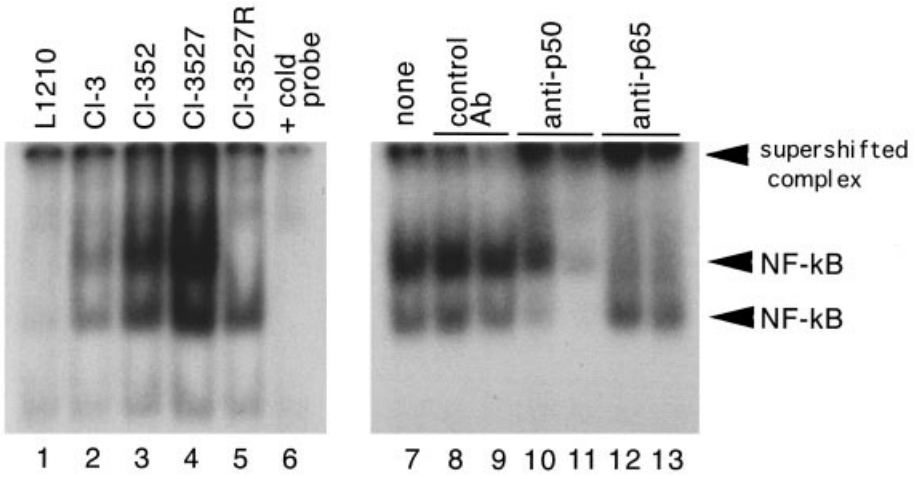

Figure 1 Electrophoretic Mobility Shift Assay (EMSA) of activated NF-κB in nuclear extracts of wild type and PARP-defective mutant L1210 cell clones

The activated NF-κB in nuclear extracts (5 μg protein) of L1210 (lane 1), Cl-3 (lane 2), Cl-352 (lane 3), Cl-3527 (lane 4) and Cl-3527R (lane 5) was assayed by EMSA, as described in the Experimental procedures section. Competition with 100-fold excess amount of cold probe (lane 6) was examined with the nuclear extract of Cl-3527. Antibody-mediated supershift analysis was carried out by using rabbit control IgG (0.5 and 1.0 μg; lanes 8 and 9, respectively), anti-p50 (0.5 and 1.0 μg; lanes 10 and 11, respectively), anti-p65 (0.25 and 0.5 μg; lanes 12 and 13, respectively) or none (lane 7), as described in the Experimental procedures section. Arrowheads indicate the two shift bands of NF-κB-DNA complexes and supershifted complexes as described in the text.

several signalling pathways [45,46], and then, the activated NF-κB complex is targeted to the nucleus. Thus, we prepared nuclear extracts from L1210, Cl-3, Cl-352 and Cl-3527 cells expressing various amounts of PARP with relative activities of 100, 48, 22 and 6%, respectively, and measured the amounts of activated NF-κB in the extracts by electrophoretic mobility shift assay (EMSA) using a specific DNA-probe containing a consensus sequence of a κB-binding site.

As, shown in Figure 1, NF-κB/DNA complexes were detected as two major shifted bands in all of the samples analysed by EMSA even without any stimulation. Interestingly, both bands showed a gradual increase with decreasing PARP content of the clones tested (lanes 1–4), suggesting an inverse correlation between PARP and NF-κB activities. Thus, Cl-3527 cells with the lowest PARP content expressed 35-fold greater activity of NF-κB than wild-type L1210 cells.

In order to characterize the two NF-κB/DNA complexes as described above, we carried out antibody-mediated supershift analysis of EMSA using specific antibodies against p50 and p65(Rel A) subunits of NF-κB, respectively. As shown in Figure 1, both bands showed supershift upon a treatment with anti-p50 (lanes 10 and 11), while only the upper band showed supershift upon a treatment with anti-p65 (lanes 12 and 13). Thus, the upper and the lower bands were demonstrated to be p50/p65 (Rel A)/DNA and p50-homodimer/DNA complexes, respectively.

To confirm further that the observed over-expression of active NF-κB is ascribed to the PARP defect, we analysed a revertant clone (Cl-3527R), which was transfected with an expression plasmid carrying a wild-type cDNA of human PARP [35]. As shown in lane 5 of Figure 1, the expression of wild-type human PARP resulted in almost complete restoration of the abnormally elevated level of p50/p65 in spite of a relatively small increase (2.3-fold) of PARP in the revertant. A relatively small decrease of approx. 50% was also detected in p50 homodimer (Figure 1, lane 5).

In order to examine whether NF-κB protein, as well as the activity, was up-regulated in PARP-deficient cells or not, we examined p65/NF-κB protein in cytosol and nuclear extracts by immunoblot analysis. As shown in Figure 2(A), the density of the immunostained band of p65 in nuclear extracts of PARP-defective Cl-352 and Cl-3527 cells was much higher than that of the wild-type cells, whereas those in cytosol remained apparently unchanged. Quantification of the relative amounts of p65, based on densitometry, revealed that nuclear extracts of Cl-352 and Cl-3527 cells contained approx. 2.2 and 7-fold of p65 of the wild-type cells, respectively, and that p65 in the cytosol of these cells showed a concomitant slight decrease (Figure 2C), suggesting that the increase of p65 in nuclear extracts was due to the translocation of the activated NF-κB from cytosol. Thus, the amount of p65 translocated to the nucleus in Cl-3527 cells was calculated to be approx. 25% of total p65 in the cells. Although we also attempted to detect p50 and IκB proteins by immunoblotting, the sensitivities of the specific antisera were insufficient for quantitative evaluation (results not shown).

Next, we examined the activated NF-κB levels in wild type L1210 cells after the treatment with 5 nM PMA, which is known to be a potent activator of NF-κB [36]. As shown in Figure 3, both NF-κB proteins, p50 homodimer and p50/p65 heterodimer, showed a maximum increase of 2 to 3-fold on EMSA after 2 h of the PMA treatment. However, even the maximum level was approx. 10-fold lower than that observed in the untreated Cl-3527 cells.

As a control experiment, we carried out EMSA for other transcription factors, SP-1 and AP-1. As shown in Figure 4, significant difference was not observed in SP-1 levels of the wild-type and the PARP-deficient clones analysed, but a relatively small increase was detected in AP-1 levels of the mutant Cl-3527.

These observations indicated that deficiency of PARP caused a marked accumulation of the activated form of NF-κB in the nucleus without affecting significantly the total NF-κB protein level.

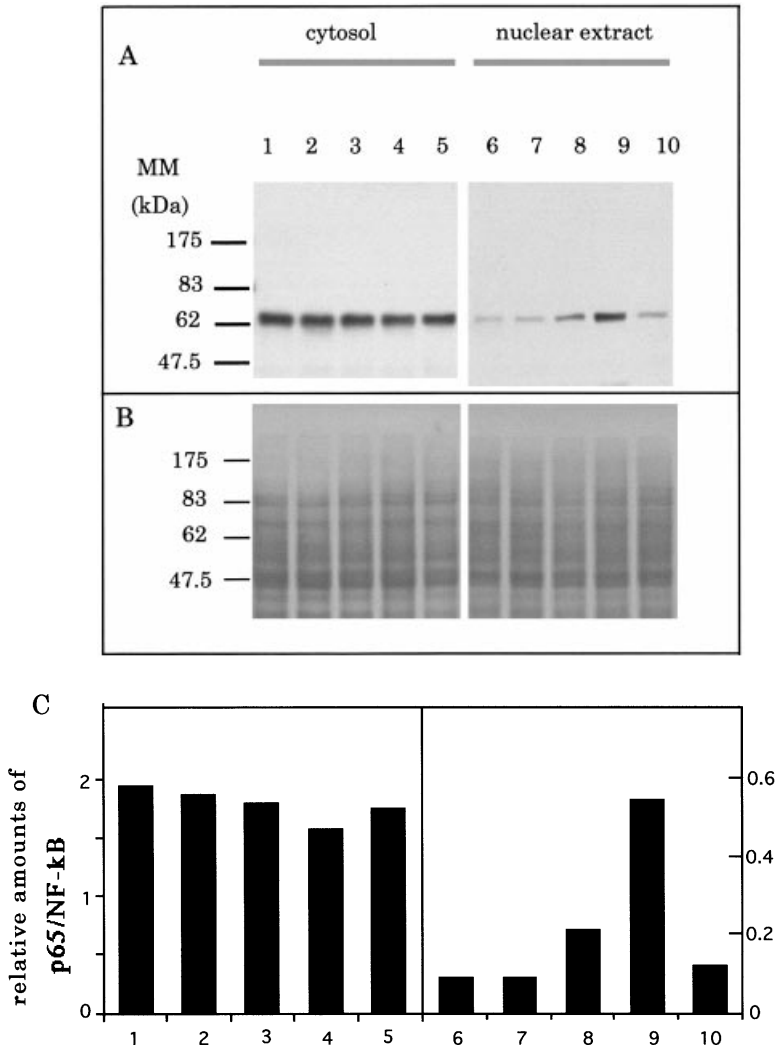

Figure 2 Immunoblot of p65/NF-κB

Cytosol and nuclear extracts of L1210 and PARP-defective mutant cells were prepared as described previously [40]. Samples of cytosol (30 μg protein) and nuclear extracts (20 μg protein) were analysed by SDS/PAGE followed by the immunoblot with anti-p65/NF-κB antibody [53] as described in the Experimental procedures section, except that Superblock® (Pierce, Rockford, Illinois, U.S.A.) was used in place of non-fat dry milk for the blocking of membrane. (**A**), Immunoblot of the cytosol (lanes 1–5) and nuclear extracts (lanes 6–10) from L1210 (lanes 1 and 6), Cl-3 (lanes 2 and 7), Cl-352 (lanes 3 and 8), Cl-3527 (lanes 4 and 9), and Cl-3527R (lanes 5 and 10) is shown. (**B**), The protein staining with Coomassie Brilliant Blue of the membrane samples used in (**A**) is shown. (**C**), The density of immunostained bands of p65 shown in (**A**) was quantified by densitometry as described in the Experimental procedures section. The values were calibrated for the number of cells used for the analysis and expressed as a relative value of the amount of p65 per 10^5 cells.

Poly(ADP-ribosyl)ation of nuclear extracts inhibits mainly the DNA-binding activity of p50/p65 heterodimer of NF-κB

An inverse correlation between cellular PARP contents and NF-κB activities as described above suggested a possibility that these two have direct interaction in cells. Thus, we examined whether poly(ADP-ribosyl)ation of nuclear extracts *in vitro* affects the DNA-binding activity of NF-κB or not. As shown in Figure 5, p50/p65/DNA complex was decreased markedly upon incubation of the nuclear extracts of Cl-3527 cells in a reconstituted PARP reaction mixture supplemented with purified PARP. The inhibition of the DNA-binding activity of NF-κB was dependent on both PARP and NAD^+, and was reversed by anti-PARP-inhibitor, 3-AB. The maximum suppression of p50/p65, attained with 1 mM NAD^+ and 0.4 μg/20 μl of PARP, was approx. 80% (compare lanes 1 and 2 with lanes 5 and 8 in Figure 5). On the other hand, poly(ADP-ribosyl)ation affected rather slightly the DNA-binding activity of p50-homodimer (approx. 40% suppression at the maximum was observed).

Since incubation of nuclear extracts with pADPR [3.6 nmol of ADP-ribose units, which corresponded to approx. 3-fold of the polymer synthesized upon poly(ADP-ribosyl)ation of nuclear extracts as described above] showed no significant effect on the DNA-binding activity of NF-κB (results not shown), it was suggested that direct ADP-ribosylation either NF-κB or its regulatory protein mediated by PARP might be involved in the suppression of NF-κB.

Poly(ADP-ribosyl)ation of purified recombinant NF-κB proteins

In order to examine whether PARP directly modifies any component of NF-κB/IκB complex, we incubated purified recombinant proteins, p50 (rp50), p65/TelA (rMBP-p65) and a

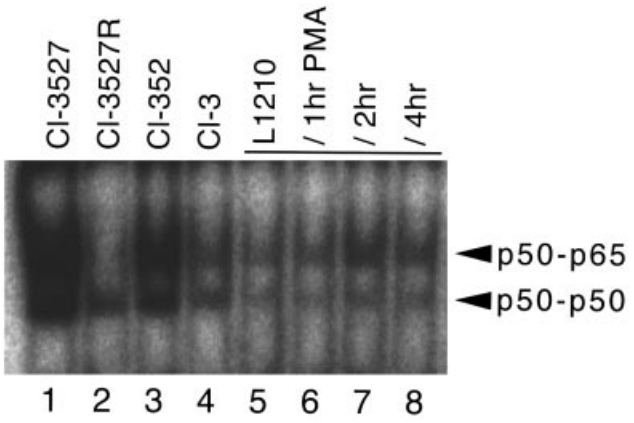

Figure 3 Activation of NF-κB by PMA treatment of L1210 cells

L1210 cells were treated with PMA (5 nM) for 1 h (lane 6), 2 h (lane 7) and 4 h (lane 8) and, then, the nuclear extracts were prepared. The NF-κB activity of nuclear extracts (10 μg protein) of the PMA-treated and untreated cells, Cl-3527 (lane 1), Cl-3527R (lane 2), Cl-352 (lane 3), Cl-3 (lane 4) and wild-type L1210 (lane 5) was assayed by EMSA.

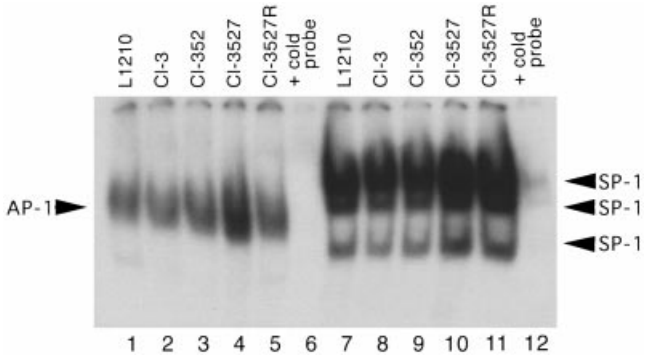

Figure 4 Effect of PARP-defect on the levels of AP-1 and SP-1 in the mutant cells

The DNA-binding activities of AP-1 (lanes 1–6) and SP-1 (lanes 7–12) in the nuclear extracts (5 μg protein) of L1210 (lanes 1 and 7), Cl-3 (lanes 2 and 8), Cl-352 (lanes 3 and 9), Cl-3527 (lanes 4 and 10) and Cl-3527R (lanes 5 and 11) were assayed by EMSA, as described in the Experimental procedures section. Competition by 100-fold excess amounts of respective cold probes (lanes 6 and 12) was examined with the nuclear extracts of Cl-3527. Arrowheads show the complexes of AP-1 and SP-1 with their specific DNA probes as indicated.

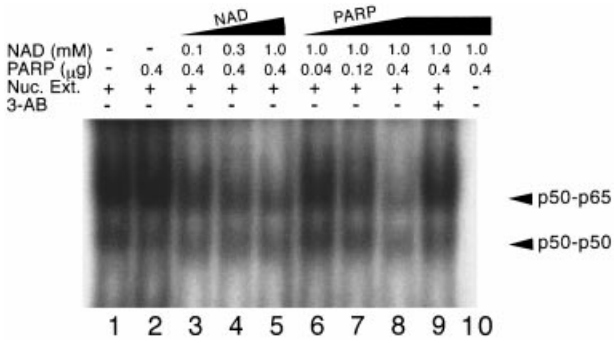

Figure 5 Poly(ADP-ribosyl)ation of nuclear extracts *in vitro* markedly suppressed DNA-binding activity of NF-κB

The nuclear extract (5 μg protein) of Cl-3527 were incubated in a poly(ADP-ribosyl)ating reaction mixture containing the indicated concentration of NAD^+ (mM) and PARP (μg/20 μl), and activated DNA (0.2 μg/20 μl) in a total volume of 50 μl of TMDT, as shown in the upper panel. Control experiments with anti-PARP-inhibitor, 3-AB (2.5 mM, lane 9) added before the enzyme reaction and without nuclear extracts (lane 10) were also carried out. After termination of the reaction with 3-AB (2.5 mM final), the activity of NF-κB in a 20 μl-aliquot (containing 2 μg proteins of nuclear extracts) of the sample was examined by EMSA. Arrowheads indicate two NF-κB/DNA complexes as described in the text.

GST-fusion protein of IκB (rGST-IκB) in a reconstituted poly(ADP-ribosyl)ating enzyme system containing [^{32}P]NAD^+ to label the modified proteins, and then, the reaction products were analysed by SDS/PAGE. As shown in Figure 6(A), both rp50 (lanes 1–3) and rMBP-p65 (lanes 5–7) were found to be poly(ADP-ribosyl)ated, whereas rGST-IκB (lane 12) and rMBP-lacZ (lane 9, included as a negative control) were not modified: p53(rMBP-p53), included as a positive control, was poly(ADP-ribosyl)ated also [47]. In this experiment, PARP reaction was carried out at a limited concentration of NAD^+ (10 μM) and in the absence of Mg^{2+} (except for lane 16) to avoid excessive and heterogeneous chain elongation of the protein-bound polymer, which is known to cause large and heterogeneous shifts of the modified proteins from the position of the unmodified one upon analysis by SDS/PAGE [41]. Thus, under the reaction condition, all of the ADP-ribosylated proteins were located close to the position of the unmodified one, except that modified PARP showed a large shift due to an extensive automodification. A PARP-inhibitor, 3AB (2.5 mM), blocked almost totally the modification of all of these proteins.

To confirm further that NF-κB proteins were covalently modified with pADPR, we carried out immunoblot analysis using an anti-pADPR antibody. As shown in Figure 7(A), all of rp50 (lane 8), rMBP-p65 (lane 2) and rMBP-p53 (lane 10) samples, which were incubated in poly(ADP-ribosyl)ating reaction mixture, were detected by immunoblotting with the antibody, confirming that these proteins were covalently modified with poly(ADP-ribose).

Effect of poly(ADP-ribosyl)ation on the DNA-binding activity of rMBP-p65 and rp50

In order to examine the effect of poly(ADP-ribosyl)ation on the function of NF-κB proteins, we carried out EMSA of recombinant NF-κB proteins. As shown in Figure 8, rMBP-p65 formed a major shift-band, which is considered to be the complex of rMBP-p65 homodimer with the DNA probe [48]. The complex formation was markedly suppressed by poly(ADP-ribosyl)ation of rMBP-p65 (lane 11) and the suppression was reversed by 3-AB (lane 12). Oligo- and poly-(ADP-ribosyl)ated PARP did not show any significant effect (lanes 8 and 9), indicating that the observed suppression was caused by a direct modification of rMBP-p65 itself.

On the other hand, incubation of rp50 in poly(ADP-ribosyl)-ating reaction mixture resulted, unexpectedly, in a slight but a significant increase of the DNA–protein complexes (Figure 8, lane 5; the lower and the upper shift-bands are considered to include a dimer and other multimers of rp50, respectively, as suggested by the manufacturer, Promega, U.S.A.). Since poly (ADP-ribosyl)ation reaction for rp50 produced a large amount of automodified PARP at the same time, we examined whether poly (ADP-ribosyl)ated PARP itself affects the DNA-binding ability of rp50. In contrast to the case of rMBP-p65, incubation of rp50 with poly(ADP-ribosyl)ated PARP resulted in a similar

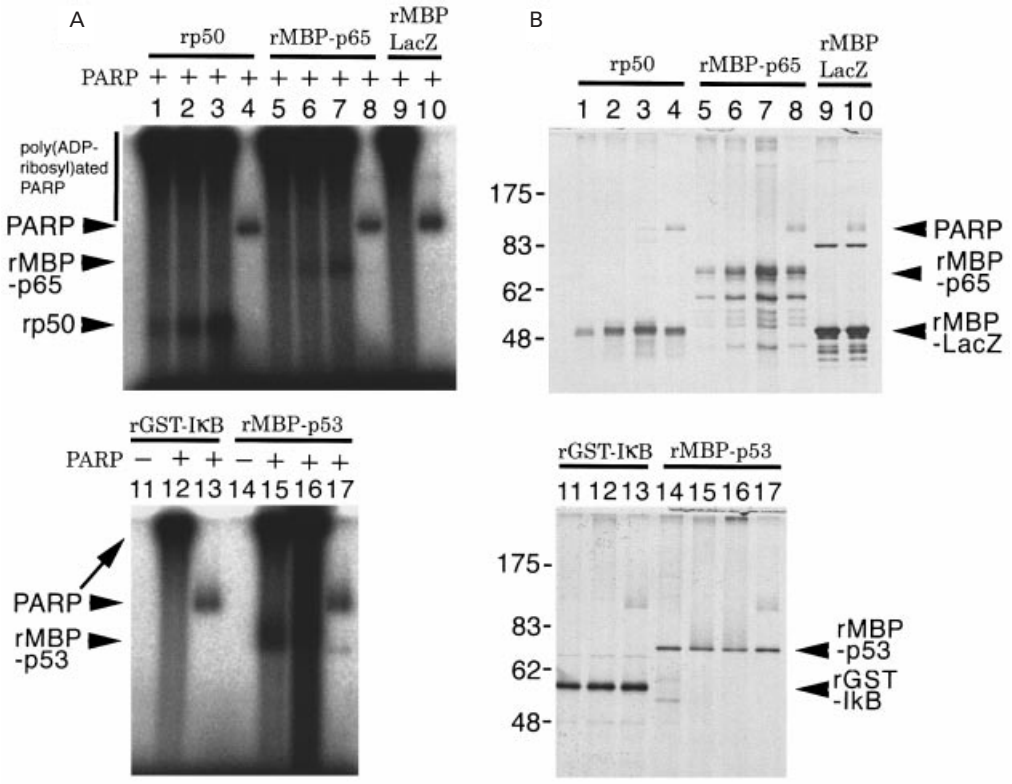

Figure 6 Poly(ADP-ribosyl)ation of purified recombinant NF-κB proteins, rMBP-p65 and rp50

NF-κB related proteins, rp50 (lanes 1–4), rMBP-p65 (lanes 5–8), and rGST-I κB (lanes 11–13), and control proteins, rMBP-LacZ (lanes 9 and 10), and rMBP-p53 (lanes 14–17), were incubated with (lanes 1–10, 12, 13, 15–17) or without (lanes 11 and 14) PARP (0.02 μg) in a 10 μl of TDT (lanes 1–15 and 17) or TMDT (lane 16) containing activated DNA (0.02 μg) and ^{32}P-NAD^{+} (1 μCi/100 pmol), as described in the Experimental procedures section. Control experiments with the addition of a PARP-inhibitor, 3-AB (2.5 mM final, lanes 4, 8, 10, 13 and 17) added before the enzyme reaction were also carried out. Recombinant proteins of 0.2 μg (lanes 1 and 5), 0.5 μg (lanes 2, 4, 6, 8, 11–17) and 1 μg (lanes 3, 7, 9 and 10) were examined for poly(ADP-ribosyl)ation. After termination of the reaction with 3-AB (2.5 mM final), the samples were separated by SDS/PAGE. The gels were silver-stained (**B**) and the ^{32}P-labelled proteins were located on a X-ray film (**A**). Arrowheads indicate the positions of the recombinant proteins and PARP.

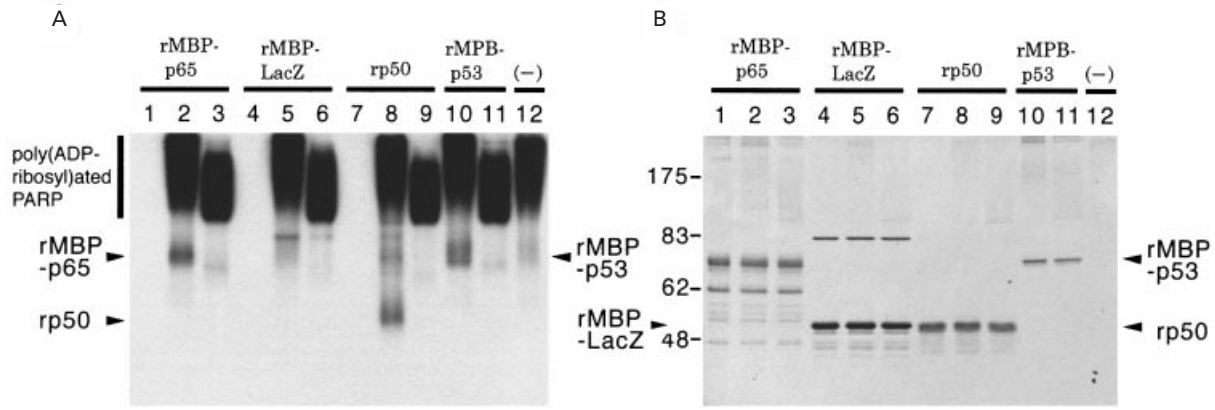

Figure 7 Immunoblot analysis of poly(ADP-ribosyl)ated recombinant NF-κB proteins with anti-poly(ADP-ribose) antibody

Two μg of rMBP-p65 (lanes 1–3), rMBP-LacZ (lanes 4–6), rp50 (lanes 7–9) and rMBP-p53 (lanes 10 and 11) were incubated in a poly(ADP-ribosyl)ating reaction mixture (TDT, 50 μl) containing activated DNA (0.1 μg) and unlabelled NAD^{+} (10 μM) in the presence (lanes 2, 3, 5, 6, 8–11) and absence (lanes 1, 4 and 7) of PARP (0.1 μg) essentially as described in Figure 5. A control experiment without recombinant proteins (lane 12) was also carried out. After termination of the reaction with 3-AB (2.5 mM final), 1/4 volume of the sample was subjected to SDS/PAGE in duplicate. One gel was silver-stained (Fig. **B**) and the other was subjected to the immunoblot analysis using appropriately diluted (1/1000) anti-pADPR antibody (**A**), as described in the Experimental procedures section. Arrowheads indicate the positions of recombinant proteins and PARP.

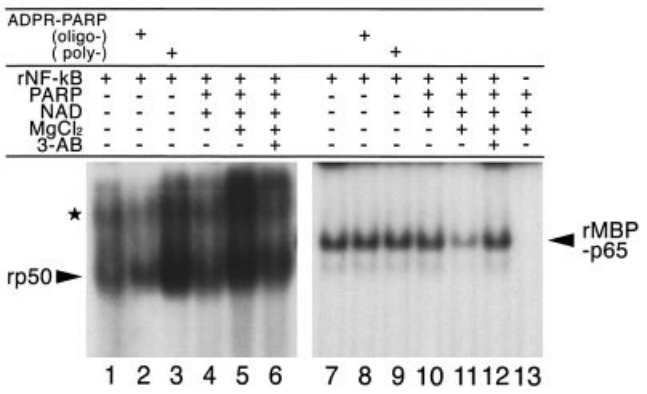

Figure 8 Effect of poly(ADP-ribosyl)ation of recombinant NF-κB proteins on their specific DNA-binding activity

NF-κB proteins rp50 (0.5 μg; lanes 1–6) and rMBP-p65 (0.5 μg; lanes 7–12) were subjected to poly(ADP-ribosyl)ation in a reaction mixture containing PARP (0.1 μg), activated DNA (0.1 μg), and NAD^+ (10 μM) in a total volume of 20 μl of TDT (lanes 1–4 and 7–10) or TMDT (lanes 5, 6, 11, 13), as described in the Experimental procedures section. In some experiments, some components of the reaction mixture were omitted as indicated in the middle panel. In other experiments, rp50 and rMBP-p65 were incubated with 0.1 μg of oligo- (lanes 2 and 8) and poly(ADP-ribosyl)ated PARP (lanes 3 and 9) instead of direct poly(ADP-ribosyl)ation of these factors, as indicated in the upper panel as ADPR-PARP. After the reaction, the DNA-binding activity of the modified recombinant protein (50 ng) was assayed by EMSA. Arrowheads indicate the complexes of an rp50 dimer and an rMBP-p65 dimer with specific DNA probe. The complex observed at the position of the asterisk (*) may be multimers of rp50 associated with the DNA probe as suggested by the manufacturer (Promega, U.S.A.).

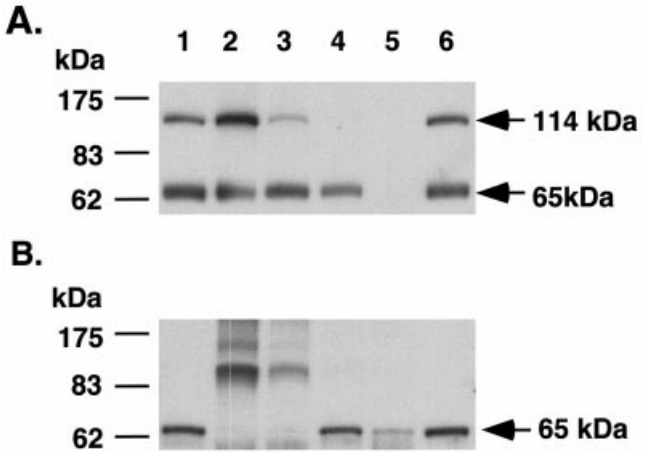

Figure 9 Immuno-detection of poly(ADP-ribosyl)ated p65/NF-κB in cytosol fraction of L1210 cells

Cytosol proteins of L1210 cells (26 μl, containing 150 μg protein) were immuno-depleted with antibody-bound Protein A-Sepharose (PAS) as follows. Rabbit control serum (lane 1), anti-p65 (lane 2), anti-PARP (lane 4), anti-pADPR (lane 5) or a mixture of an equal volume of anti-p65 and anti-PARP antibodies (lane 3). A control sample treated with PAS only was also included (lane 6). The unbound fractions were collected and analysed by SDS/PAGE (10% gel) according to the method of Laemmli [54], followed by immunoblotting with anti-pADPR (**A**) and anti-p65 (**B**) antibodies.

extent of activation of the DNA-binding ability of rp50 (Figure 8, lane 3) as observed in the direct modification reaction (lane 5), suggesting that the observed activation was caused by poly(ADP-ribosyl)ated PARP itself.

Demonstration of poly(ADP-ribosyl)ation of p65/NF-κB *in vivo*

In order to examine poly(ADP-ribosyl)ation of NF-κB *in vivo*, we carried out immunoblot of nuclear and cytosol fraction from wild-type L1210 cells with anti-pADPR antibody. Since approx. 80% of poly(ADP-ribosyl)ated proteins were detected in the cytosol fraction (results not shown), we examined whether NF-κB is poly(ADP-ribosyl)ated *in vivo* using the cytosol fraction of wild type and mutant Cl-3527 cells. Two major and seven minor poly(ADP-ribosyl)ated proteins were detected in the cytosol fraction of wild-type cells, whereas almost all of these bands were absent from the cytosol of Cl-3527 cells (results not shown). Thus, we carried out immuno-precipitation for L1210 cytosol with anti-p65, anti-pADPR and anti-PARP antibodies. Since the trial to detect p65 in the immuno-precipitates was unsuccessful due to the disturbance by serum-derived contaminants, we analysed unbound fractions by immunoblotting with anti-pADPR (Figure 9A) and anti-p65/NF-κB (Figure 9B) antibodies.

As shown in Figure 9(A), control samples (lanes 1 and 6) contained two major poly(ADP-ribosyl)ated proteins, which were located at around 114 kDa and 65 kDa, respectively. The 114 kDa band was poly- (or probably oligo-) ADP-ribosylated PARP since immuno-precipitation with either anti-PARP (lane 4) or anti-pADPR (lane 5) antibody depleted the band. This 65 kDa band, upon immuno-precipitation with anti-p65, showed a slight but significant decrease (about 15% decrease was repeatedly observed). Interestingly, the intensity of this 65 kDa band was also significantly decreased upon immuno-precipitation with anti-PARP antibody (lane 4). In Figure 9(B), where a parallel run of the samples in Figure 9(A) was immunostained with anti-p65 antibody, the intensity of 65 kDa band was significantly diminished by immuno-depletion with anti-pADPR antibody (in lane 5, p65 subunit of NF-κB was decreased by approx. 60%) or with anti-PARP (in lane 4, the intensity was decreased by 15%). Immuno-depletion of p65 by anti-pADPR or anti-PARP antibodies was observed reproducibly. These findings suggest that PARP might interact with p65 and modify p65 by poly(ADP-ribosyl)ation.

DISCUSSION

There are many reports suggesting that PARP is involved in the control of gene expression. Although the exact mechanism has not been fully elucidated, recent studies from several laboratories revealed that several transcription factors such as TEF-1 [34], TFIIF [33], YY1 and TBP [44] were poly(ADP-ribosyl)ated by PARP *in vitro*. It is also reported that transcription factors Oct-1 [43] and TEF-1 [34] form stable complexes with PARP.

In the present report, we demonstrated: (i) nuclear extracts of PARP-deficient L1210 cell clones showed extremely high DNA-binding activity of NF-κB, and the PARP contents and active NF-κB levels in the mutant cells were inversely correlated (Figure 1); (ii) judging from the immunoblotting of p65/NF-κB in cytosol and nuclear extracts, the increase of NF-κB DNA binding in PARP-deficient L1210 cells was mainly due to the activation rather than the increase in the expression of NF-κB protein (Figure 2); (iii) poly(ADP-ribosyl)ation of nuclear extracts *in vitro* markedly suppressed the DNA-binding activity of NF-κB proteins, especially a heterodimer, p50/p65 (RelA) (Figure 5); (iv) both recombinant NF-κB proteins, rMBP-p65 and rp50, but not rGST-IκB, were covalently modified by PARP (Figures 6

and 7); (v) poly(ADP-ribosyl)ation of rMBP-p65 resulted in a marked decrease in its ability to form a complex with the specific DNA probe (Figure 8), and (vi) p65 was poly(ADP-ribosyl)ated *in vivo* at least in a part. All of these results strongly suggest a possibility that poly(ADP-ribosyl)ation is involved in the regulation of NF-κB, which is known to be a specific transcription factor involved in the control of numerous genes, including those of many cellular genes involved in immune responses and inflammation as well as some virus genes [36].

Recent studies on NF-κB have revealed that various extracellular stimuli are transmitted to cytosol through several signalling pathways involving various protein kinases [45,46] and result in the degradation and release of IκB from an inactive NF-κB/IκB complex, which promotes the translocation of the activated p50/p65 complex to the nucleus [36]. So far, we have not enough evidence to explain clearly how PARP affects the fundamental signalling pathway of NF-κB or its metabolism in L1210 cells. However, the present results suggest that PARP and poly(ADP-ribosyl)ation play a role in re-localization of nuclear p65/NF-κB to cytoplasm in addition to the possible effect on NF-κB-dependent transcription. Thus, accumulation of the activated p65 in Cl-3527 nuclei may be due to the defect in nuclear exportation and cytoplasmic re-localization caused by PARP-deficiency. In this regard, it is interesting that the nuclear extracts of Cl-3527 cells contained approx. 3-fold higher amounts of poly(ADP-ribosyl)ated 65 kDa protein compared with that of wild-type L1210 (K. Yoshihara, unpublished work).

During the preparation of this manuscript Oliver et al. [49] and Hassa and Hottiger [50] reported that PARP is required for the NF-κB-dependent transcriptional activation process by utilizing PARP(PARP-1)-gene disrupted mice or cells. Their results accord well with our previous observation that the NF-κB-dependent signalling is markedly suppressed in a PARP-deficient L-1210 clone, Cl-3527 [51]. However, a discrepancy seems to exist with the PARP-1-gene disrupted cells and Cl-3527, since the former does not seem to accumulate p65/NF-κB in nuclei before the cells are stimulated by an agent to activate NF-κB [49,50]. The discrepancy observed in the two PARP-defective cells may be due to the difference in the residual poly(ADP-ribosyl)ating activity in these mutants.

Although the influence of accumulation of p65/NF-κB in nuclei on the physiology of cells has not been fully studied, the mutant showed an elongated doubling time and a higher temperature sensitivity at 42 °C compared with those of wild-type cells [35], while all of them showed a stable, exponential growth at 33 and at 37 °C. These features of PARP mutant cells could be ascribed to the abnormal NF-κB level either directly or indirectly.

Considering various biological roles of NF-κB in transcriptional regulation and prevention of apoptosis [52], the present observation may provide a novel model for regulation of cellular function by poly(ADP-ribosyl)ation.

We thank Dr. B. Vogelstein (Johns Hopkins Oncology Center, U.S.A.) for the gift of the cDNA for wild-type p53. We also thank the Japanese Cancer Research Resources Bank (National Institute of Hygienic Science, Tokyo) for the donation of a cell line, L1210. This work was supported in part by a Grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture.

REFERENCES

1 Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., Takeda, M. and Hayaishi, O. (1969) Cold Spring Harbor Symp. Quant. Biol. **34**, 781–786
2 Tanuma, S. and Johnson, G. S. (1983) J. Biol. Chem. **258**, 4067–4070
3 Yoshihara, K., Tanigawa, Y., Burzio, L. and Koide, S. S. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 289–293
4 Tanaka, Y., Yoshihara, K., Itaya, A., Kamiya, T. and Koide, S. S. (1984) J. Biol. Chem. **259**, 6579–6585
5 Ferro, A. M. and Olivera, B. M. (1984) J. Biol. Chem. **259**, 547–554
6 Schroder, H. C., Steffen, R., Wenger, R., Ugarkovic, D. and Muller, W. E. (1989) Mutat. Res. **219**, 283–294
7 Creissen, D. and Shall, S. (1982) Nature (London) **296**, 271–272
8 Yoshihara, K., Itaya, A., Tanaka, Y., Ohashi, Y., Ito, K., Teraoka, H., Tsukada, K., Matsukage, A. and Kamiya, T. (1985) Biochem. Biophys. Res. Commun. **128**, 61–67
9 Tanaka, Y., Ito, K., Yoshihara, K. and Kamiya, T. (1986) Eur. J. Biochem. **155**, 19–25
10 Ohashi, Y., Itaya, A., Tanaka, Y., Yoshihara, K., Kamiya, T. and Matsukage, A. (1986) Biochem. Biophys. Res. Commun. **140**, 666–673
11 Yoshihara, K., Hashida, T., Yoshihara, H., Tanaka, Y. and Ohgushi, H. (1977) Biochem. Biophys. Res. Commun. **78**, 1281–1288
12 Yoshihara, K., Hashida, T., Tanaka, Y., Matsunami, N., Yamaguchi, A. and Kamiya, T. (1981) J. Biol. Chem. **256**, 3471–3478
13 Durkacz, B. W., Irwin, J. and Shall, S. (1981) Biochem. Biophys. Res. Commun. **101**, 1433–1441
14 Trucco, C., Oliver, F. J., de Murcia, G. and Menissier-de Murcia, J. (1998) Nucleic Acids Res. **26**, 2644–2649
15 Yoshida, S. and Simbulan, C. M. (1994) Mol. Cell. Biochem. **138**, 39–44
16 Simbulan-Rosenthal, C. M., Rosenthal, D. S., Hamid Boulares, A., Hickey, R. J., Malkas, L. H., Coll, J. M. and Smulson, M. E. (1998) Biochemistry **37**, 9363–9370
17 Magnusson, J. and Ramel, C. (1990) Mutagenesis **5**, 511–514
18 Berger, N. A., Sims, J. L., Catino, D. M. and Berger, S. J. (1983) Princess Takamatsu Symp. **13**, 219–216
19 Yamamoto, H., Uchigata, Y. and Okamoto, H. (1981) Nature (London) **294**, 284–286
20 Okamoto, H. and Yamamoto, H. (1983) Princess Takamatsu Symp. **13**, 297–308
21 Hayaishi, O. and Ueda, K. (1982) ADP-ribosylation Reactions: Biology and Medicine, Academic Press, N.Y.
22 de Murcia, G., Jacobson, M. and Shall, S. (1995) Trends Cell Biol. **5**, 78–81
23 Lindahl, T., Satoh, M. S., Poirier, G. G. and Klungland, A. (1995) TIBS **20**, 405–411
24 Ménissier de Murcia, J., Niedergang, C., Trucco, C., Ricoul, M., Dutrillaux, B., Mark, M., Oliver, F. J., Masson, M., Dierich, A., LeMeur, M., Walztinger, C., Chambon, P. and de Murcia, G. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 7303–7307
25 Wang Zhao-Qi, Sting, L., Morrison, C., Jantsch, M., Los, M., Schulze-Osthoff, K. and Wagner, E. F. (1997) Genes and Development **11**, 2347–2358
26 Elliasson, M. J. L., Sampei, K., Mandir, A. S., Hurn, P. D., Traystman, R. J., Bao, J., Pieper, A., Wang, Zhao-Qi, Dawson, T. M., Snyder, S. H. and Dawson, V. L. (1997) Nature Medicine **3**, 1089–1095
27 Burkart, V., Wang, Zhao-Qi, Radons, J., Heller, B., Herceg, Z., Stingl, L., Wagner, E. F. and Kolb, H. (1999) Nature Medicine **5**, 314–319
28 Masutani, M., Suzuki, H., Kamada, N., Watanabe, M., Ueda, O., Nozaki, T., Jishage, K., Watanabe, T., Sugimoto, T., Nakagama, H., Ochiya, T. and Sugimura, T. (1999) Proc. Natl. Acad. Sci. U.S.A. **96**, 2301–2304
29 Wang, Zhao-Qi, Auer, B., Sting, L., Berghammer, H., Haidacher, D., Schweiger, M. and Wagner, E. F. (1995) Genes and Development **9**, 509–520
30 Amé, H-C., Rolli, V., Schreiber, V., Niedergang, C., Apiou, F., Decker, P., Muller, S., Höger, T., Ménissier-de Murcia, J. and de Murcia, G. (1999) J. Biol. Chem. **274**, 17860–17868
31 Qu, Z., Fujimoto, S. and Taniguchi, T. (1994) J. Biol. Chem. **269**, 5543–5547
32 Meisterernst, M., Stelzer, G. and Roeder, R. G. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 2261–2265
33 Rawling, J. M. and Alvares-Gonzalez, R. (1997) Biochem. J. **324**, 249–253
34 Butler, A. J. and Ordahl, C. P. (1999) Mol. Cell Biol. **19**, 296–306
35 Yoshihara, K., Itaya, A., Hironaka, T., Sakuramoto, S., Tanaka, Y., Tsuyuki, M., Inada, Y., Kamiya, T., Ohnishi, K., Honma, M. et al. (1992) Exp. Cell Res. **200**, 126–134
36 Ghosh, S., May, M. J. and Kopp, E. B. (1998) Annu. Rev. Immunol. **16**, 225–260
37 Uoshihara, K., Hashida, T., Tanaka, Y., Ohgushi, H., Yoshihara, H. and Kamiya, T. (1978) J. Biol. Chem. **253**, 6459–6466
38 Yoshihara, K., Tanaka, Y., Yoshihara, H., Hashida, T., Ohgushi, H., Arai, R. and Kamiya, T. (1980) in Novel ADP-ribosylations of Regulatory Enzymes and Proteins, Developments in Cell Biology, 6th edn., (Smulson, M. E. and Sugimura, T., eds), pp. 33–44, Elsevier/North-Holland, New York, Amsterdam, Oxford
39 Shima, T., Fujimura, S., Hasegawa, S., Shimizu, Y. and Sugimura, T. (1970) J. Biol. Chem. **245**, 1327–1330
40 Tobiume, M., Fujinaga, K., Kameoka, M., Kimura, T., Nakaya, T., Yamada, T. and Ikuta, K. (1998) J. Gen. Virol. **79**, 1363–1371
41 Tanaka, Y., Yoshihara, K., Ohashi, Y., Itaya, A., Nakano, T., Ito, K. and Kamiya, T. (1985) Anal. Biochem. **145**, 137–143
42 Kameoka, M., Kimura, T., Zheng, Y.-H., Suzuki, S., Fujinaga, K., Luftig, R. B. and Ikuta, K. (1997) J. Clin. Microbiol. **35**, 41–47

43 Nie, J., Sakamoto, S., Song, D., Qu, Z., Ota, K. and Taniguchi, T. (1998) FEBS Lett. **424**, 27–32
44 Oei, S. L., Griesenbeck, J., Schweiger, M. and Ziegler, M. (1998) J. Biol. Chem. **273**, 31644–31647
45 Béraud, C., Henzel, W. and Baeuerle, P. A. (1999) Proc. Natl. Acad. Sci. U.S.A. **96**, 429–434
46 Spencer, W., Kwon, H., Crépieux, P., Leclerc, N., Lin, R. and Hiscott, J. (1999) Oncogene **18**, 495–505
47 Agarwal, M. L., Agarwal, A., Taylor, W. R., Wang, Zhao-Qi, Wagner, E. F. and Stark, G. R. (1997) Oncogene **15**, 1035–1041
48 Fujita, T., Nolan, G. P., Ghosh, S. and Baltimore, D. (1992) Genes Dev. **6**, 775–787
49 Oliver, F. J., Ménissier de Murcia, J., Nacci, C., Decker, P., Andriantsitohaina, R., Muller, S., de la Rubia, G., Stoclet, C. and de Murcia, G. (1999) EMBO J. **18**, 4446–4454
50 Hassa, P. O. and Hottiger, M. O. (1999) Biol. Chem. **380**, 953–959
51 Kameoka, M., Tanaka, Y., Ota, K., Itaya, A. and Yoshihara, K. (1999) Biochem. Biophys. Res. Commun. **262**, 285–289
52 Liu, Z. G., Hsu, H., Goeddel, D. V. and Karin, M. (1996) Cell **87**, 565–576
53 Hayashi, T., Sekine, T. and Okamoto, T. (1993) J. Biol. Chem. **269**, 26790–26795
54 Laemmli, U. K. (1970) Nature **227**, 680–685

Received 6 July 1999/26 November 1999; accepted 22 December 1999