The efficient repair of DNA double-strand breaks (DSBs) is critical for the maintenance of genomic integrity. In mammalian cells, the nonhomologous end-joining process that represents the predominant repair pathway relies on the DNA-dependent protein kinase (DNA-PK) and the XRCC4-DNA ligase IV complex. Nonetheless, several in vitro and in vivo results indicate that mammalian cells use more than a single end-joining mechanism. While searching for a DNA-PK-independent end-joining activity, we found that the pretreatment of DNA-PK-proficient and -deficient rodent cells with an inhibitor of the poly(ADP-ribose) polymerase-1 enzyme (PARP-1) led to increased cytotoxicity of the highly efficient DNA double-strand breaking compound calicheamicin $\gamma_1$. In addition, the repair kinetics of the DSBs induced by calicheamicin $\gamma_1$ was delayed both in PARP-1-proficient cells pretreated with the PARP-1 inhibitor and in PARP-1-deficient cells. In order to get new insights into the mechanism of an alternative route for DSB repair, we have established a new synopsis and end-joining two-step assay in vitro, operating on DSBs with either nuclear protein extracts or recombinant proteins. We found an end-joining activity independent of the DNA-PK/XRCC4-ligase IV complex but that actually required a novel synopsis activity of PARP-1 and the ligation activity of the XRCC1-DNA ligase III complex, proteins otherwise involved in the base excision repair pathway. Taken together, these results strongly suggest that a PARP-1-dependent DSBs end-joining activity may exist in mammalian cells. We propose that this mechanism could act as an alternative route of DSBs repair that complements the DNA-PK/XRCC4/ligase IV-dependent nonhomologous end-joining.

DNA double-strand breaks (DSBs) represent normal intermediates during physiological processes like meiosis or V(D)J recombination but also toxic lesions produced by collapsed DNA replication forks and by DNA-damaging agents such as ionizing radiation (IR) or reactive oxygen species. Repair of DSBs is critical for the maintenance of genomic integrity because improperly repaired breaks can lead to cancer via chromosomal aberrations (1, 2).

In eukaryotic cells, DSBs are repaired through two major pathways: homologous recombination (HR) and nonhomologous end-joining (NHEJ) (for reviews see Refs. 3–6). It is largely admitted that DSBs are repaired by NHEJ at least in the G1 phase of the cell cycle, whereas HR operates in late S/G2 (7). The NHEJ process requires several factors that recognize and bind the double-strand break, catalyze the synopsis of the broken ends, and then process and reseal the break (8, 9). The NHEJ pathway relies on a set of proteins comprising at least (i) a DNA end binding activity, the DNA-dependent protein kinase (DNA-PK) that consists of the catalytic subunit DNA-PKcs and the Ku70/Ku80 heterodimer (10, 11), and (ii) a DNA break resealing activity, the XRCC4-DNA ligase IV complex (12, 13).

In order to gain insight into the NHEJ mechanism, both in vitro and in vivo approaches have been undertaken. The end-joining reaction has been studied in vitro by various assays, many of them using incubation with cell extracts of plasmid DNA linearized by enzymatic restriction as a model for DSBs containing substrates (17). Results of these in vitro repair experiments have brought substantial evidence for an alternative DNA-PK-independent end-joining pathway (18–24). In vivo studies also support the hypothesis that several DNA end-joining pathways exist either in yeast (25, 26) or in mammalian cells (27–31). For example, end-joining of restricted DNA remained efficient after transfection of cells deficient in various components of the DNA-PK-dependent pathway and relied mostly on an end homology-dependent mechanism (29). Also, it was shown recently that Ku was dispensable for the microhomology-directed rejoining of an intrachromosomal DSB (32). Taken together, these in vitro and in vivo studies strongly suggest that end-joining of DSBs relies on more than the DNA-PK-dependent pathway.

Any candidate for an alternative end-joining pathway of DSBs would recognize DNA ends, and its defect would have an impact on cell survival to double-strand breaking agents. Based on its high binding affinity for DNA DSBs (33, 34), the involvement of poly(ADP-ribose) polymerase-1 protein (PARP-1) in sensing and/or repairing DSBs can be hypothesized. PARP-1 is involved in different cellular processes, in-
including the DNA base excision repair pathway (BER), responsible for the removal of alkylated bases and abasic sites (for reviews see Refs. 35–38). PARP-1 catalyzes the covalent transfer of successive units of ADP-ribose moiety from NAD to itself and other nuclear protein acceptors in a manner dependent upon the presence of DNA single-strand breaks (SSBs). A possible role of PARP-1 in the repair of DSBs is supported by the following points. (i) Ku heterodimer does not represent the sole DSBs recognition complex in cell extracts because it was originally pointed out that PARP-1 is activated in vitro not only by SSBs but also by DSBs (39) and that purified PARP-1 binds to DSBs with an efficacy higher than that to SSBs (33, 34) and with an affinity even greater than that of DNA-PK (34). (ii) PARP-1 has been shown to interact with both subunits of DNA-PK (40, 41) and to catalyze their poly(ADP-ribosyl)ation (42, 43). (iii) In cells, nuclear areas of poly(ADP-ribosylation) were induced concomitantly to the formation of direct DSBs via V(D)J recombination in the absence of functional DNA-PK (44). (iv) DSB repair was delayed under conditions of overexpression of the catalytically inactive DNA-binding domain of PARP-1 (45) or when cells were pretreated with a PARP-1 inhibitor (46–50). Despite all these data, no biochemical experiments were yet specifically devoted to substantiate the potential role of PARP-1 in the repair of DSBs.

Classical DSB-inducing agents such as IR mostly induce SSBs and base damage but only a few percent of DSBs. Therefore, we have used calicheamicin γ1 that is a more potent producer of DSBs (51). We initially found that both chemical PARP inhibition or genetic loss of PARP-1 function impaired DSB repair. Then we developed a DNA pull-down assay using nuclear extracts (NE) from mammalian cells. By using this approach, we have identified a new synapsis and end-joining activity independent of the classical NHEJ. Proteins that we have reconstituted this DSB end-joining activity with recombinant PARP-1, XRCC1, and DNA ligase III, proteins otherwise in reconstituted this DSB end-joining activity with recombinant PARP-1 (52) or when cells were pretreated with a PARP-1 inhibitor (46–50).

Despite all these data, no biochemical experiments were yet specifically devoted to substantiate the potential role of PARP-1 in the repair of DSBs. In all these cases, though, we have observed that PARP-1-dependent DNA end-joining activity with recombinant PARP-1, XRCC1, and DNA ligase III, proteins otherwise involved in BER (52). Taken together, these results suggest that a PARP-1-dependent DSB end-joining mechanism operates in cells as an alternative route that the DNA-PK/C/44 ligase IV-dependent NHEJ.
Adenylation Assay—After incubation with nuclear extracts as above, DNA beads were reacted for 1 h at 30 °C under gentle agitation in reaction buffer containing 5 μCi of [α-32P]yATP (3000 Ci/μmol, ICN Pharmaceuticals, Inc.). After five washes in reaction buffer, DNA beads were heated in SDS sample buffer and resolved by 8% SDS-PAGE followed by electrophoretic transfer onto polyvinylidene difluoride membrane (Amersham Biosciences). Adenylated proteins were detected by autoradiography of the membrane processed with a PhosphorImager (Storm System TM, Amersham Biosciences).

DNA Rejoining Assay with Purified and Recombinant Proteins—The vector coding for the histidine-tagged recombinant human DNA ligase III was a kind gift from Dr. T. Lindahl and Dr. D. Barnes, and the protein was produced as described (55). Recombinant human XRCC1 was provided by J. P. Radicella (CEA, Fontenay-aux-Roses, France) and was produced as described (56). Human recombinant PARP-1 protein (95% purity) was from Trevigen (Gaithersburg, MD). For the reaction with purified recombinant proteins PARP-1, XRCC1, and DNA ligase III were preincubated in reaction buffer B (20 mM Tris, pH 7.5, 10 mM MgCl₂, 60 mM KOAc, 0.5 mM dithiothreitol, 0.3 mg/ml bovine serum albumin) at 30 °C for 15 min prior to 30 min of incubation with DNA beads at 30 °C, and the reaction was then conducted in reaction buffer B as in the DNA pull-down assay with nuclear extracts.

RESULTS

Effect of PARP-1 Inhibition on the Cytotoxicity of Calicheamicin γ1—IPARP-1 is involved in an end-joining reaction in vivo, a specific inhibitor should potentiate the cytotoxic effect of a DSB inducer. Indeed, previous reports (48–50) have shown such potentiation effect when IR was used in combination with a PARP-1 inhibitor. However, IR produce mainly SSBs, despite the fact that DSBs are believed to be responsible for cell toxicity (57). Consequently, we used calicheamicin γ1 as a DSB generator. This natural hydrophobic enediyne antibiotic has been shown to produce DSBs with selectivity and efficiency higher than IR, yielding a 1:3 ratio of DNA DSBs to SSBs in vivo, compared with a 1:20 ratio for IR (51). In addition, we anticipated that the sensitization effect by a PARP inhibitor might rely on an end-joining reaction independent of DNA-PK. Therefore, we investigated by clonogenic assay the sensitivity of DNA-PK-proficient and -deficient rodent cells to the radiomimetic compound calicheamicin γ1, in the presence or absence of a nontoxic concentration of the potent PARP-1 inhibitor DIQ (58). We treated in parallel two paired CHO cell lines, the control AA8 and DNA-PKcs-deficient V3 cell lines (59), and the hamster control Ku80-DNA-complemented xrs6(xrs6/haKu80) and the xrs6 Ku-deficient cell lines (60).

First, we confirmed that DIQ was a potent inhibitor of PARP-1 activity in vivo (61, 62) since we observed a dose-dependent inhibition by DIQ of the H2O2-induced overall cellular protein poly(ADP-ribose)ylation by Western blotting with an anti-PAR antibody (data not shown). By performing clonogenic assay on the four rodent cell lines, we measured the cytotoxicity of DIQ and chose 10 μM as the highest drug concentration that still allowed 100% cell survival (data not shown). Then we measured the clonogenic survival to calicheamicin γ1 of rodent cells pretreated or not with 10 μM DIQ. Fig. 1, A and B, showed that DNA-PK deficiency sensitizes cells to calicheamicin γ1 as compared with the respective control, 5.5-fold for xrs6 and 3.5-fold for V3 cells at LD₅₀ (concentration of drug that inhibited cell survival by 50%), as already reported (63). To assess the effect of PARP-1 inhibition on cell sensitivity, we calculated for each cell line the potentiating factor as the mean ratio of LD₅₀ for calicheamicin γ1 alone divided by LD₅₀ in the presence of DIQ. As shown in Table I, all the ratios were significantly superior to 1, clearly indicating that DIQ potentiates the cytotoxicity of calicheamicin γ1 in both DNA-PK-proficient and -deficient cells.

Kinetics of DSB Repair Determined by H2AX Dephosphorylation—Although calicheamicin γ1 induces DSBs at a rate higher than IR, it also produces SSBs. Thus, it was necessary to focus on the repair of DSBs. It has been shown recently that a PARP inhibitor decreased DNA DSB repair in both DNA-PK-proficient and -deficient CHO cells exposed to IR (48, 50). Because DSB repair was quantified by neutral filter elution in these reports, we thought to use an alternative method in order to assess DSBs repair under similar conditions. Recent studies have demonstrated that the intracellular content of the phosphorylated histone variant H2AX (named γ-H2AX) can be used to measure the repair of DSBs in CHO cells at low yield of DSBs (1 gray (7)). Under experimental conditions similar to ours, it has been calculated that calicheamicin γ1 produced about 400 DSBs/nm (51). Accordingly, we chose a drug dose (3 μM) corresponding to the LD₁₀ for xrs6/haKu80 cells as measured by clonogenic assay (Fig. 1). Then we followed the kinetics...
of loss of γ-H2AX phosphorylation after a 1-h treatment of both DNA-PK-proficient and -deficient cells with this concentration of calicheamicin γ1, in the presence or absence of DIQ. Proliferating cell nuclear antigen was used as a control of protein loading. As shown in Fig. 2A, γ-H2AX was detected in whole cells extracts only after treatment of both DNA-PK-proficient and -deficient cells with the same dose of calicheamicin γ1 (Fig. 2A, compare lanes 1 and 3). In the absence of DIQ, only DNA-PK-proficient cells showed a kinetics of γ-H2AX dephosphorylation over the 1-h post-treatment period examined (Fig. 2A, compare lanes 3 and 9 for xrs6/haKu80 and xrs6 cell lines), indicating that it is directly correlated to the cellular DSB repair activity. In addition, the half-time of γ-H2AX loss is generally longer than the DSB rejoining half-time (64). This could explain that phosphorylated H2AX was still detected 1-h post-treatment even in DNA-PK-proficient cells (Fig. 2A, lane 9 for xrs6/haKu80). In the presence of DIQ, there was an obvious potentiation of γ-H2AX phosphorylation already detected at time point 0 post-treatment, in both cell lines (Fig. 2A, compare lanes 3 and 4). This observation is compatible with the possibility that a PARP-1-dependent DSBs repair pathway already takes place within the 1-h time interval of the drug treatment. Additionally, H2AX phosphorylation was maintained over the 1-h-post-treatment period in both cell lines in the presence of DIQ (Fig. 2A, compare lanes 4 and 10).

These results support the possibility that PARP-1 is involved in the early repair of a significant fraction of DNA DSBs that escape DNA-PK-dependent end-joining. However, DIQ could have an effect on γ-H2AX dephosphorylation independently of DSB repair. In addition, PARP-1 inhibition could block the enzyme on DNA breaks and impair their repair by conventional mechanisms, rather than preventing PARP-1 to participate in a PARP-dependent repair route. In order to check these possibilities, we followed the kinetics of loss of γ-H2AX phosphorylation in both PARP-1-proficient and -deficient MEF cells after a short treatment with calicheamicin γ1, in the presence or absence of DIQ. As shown in Fig. 2B, the kinetics of γ-H2AX dephosphorylation in PARP-1-proficient cells was similar to that observed in xrs6/haKu80 (compare lanes 3 and 9 in Fig. 2B, upper panel, and in Fig. 2A, upper panel). In PARP-1−/− cells, there is an obvious potentiation of γ-H2AX phosphorylation already detected at time point 0 post-treatment as compared with PARP-1-proficient cells (Fig. 2B, upper panel, compare lanes 3 and 4), and this difference was maintained all over the post-treatment period examined (Fig. 2B, upper panel, compare lanes 9 and 10). In the presence of DIQ, there was no observable effect on the kinetics of loss of γ-H2AX phosphorylation in PARP-1−/− cells (Fig. 2B, compare lanes 6, 8 or 10 in the lower panel with the same lanes in the upper panel); in contrast, DIQ slowed down the kinetics of γ-H2AX dephosphorylation in the PARP-1-proficient cells (Fig. 2B, compare lanes 7 or 9 in the lower panel with the same lanes in the upper panel) that then became indistinguishable with the PARP-1−/− cells (Fig. 2B, lower panel, compare lanes 9 and 10).

These results confirm recent reports (48) that indicate a role of PARP-1 activity in cell survival to DSBs. Moreover, cell sensitization to calicheamicin γ1 treatment using PARP-1 inhibitor paralleled the effect on DSBs repair, as determined by the kinetics of γ-H2AX dephosphorylation. These results suggest that the inhibition of a PARP-dependent route for DSBs repair by DIQ could play a role in its sensitization effect toward DSBs generators.

Characterization of a Mammalian End-joining Activity via a Two-stage DNA Pull-down Assay—Because a DSB repair mechanism independent of DNA-PK but relying on PARP-1 activity might contribute to cell survival, we decided to set up an in vitro assay in order to test the involvement of PARP-1 in an end-joining activity. A biochemical assay dependent on the presence of DNA-PK and XRCC4/ligase IV has already been reported (65). Because the end-joining process dependent on DNA-PK most probably requires a synapsis reaction between proteins present at each DNA end (66), we deliberately unfavorable such reaction by using a two-step in vitro DNA pull-down assay with NEs from mammalian cells (Fig. 3). We used a 30-bp double-stranded DNA fragment (YC) biotinylated on one 5′-end and bearing a 5′-phosphate group on the opposite end allowing ligation between two YC fragments. In a first step, YC bound to streptavidin-conjugated magnetic beads (YC beads) was incubated with HeLa NEs, allowing recruitment of protein complexes recognizing DNA ends, namely at least DNA-PK and PARP-1. After a wash, the same oligonucleotide radiolabeled on the 5′-end (YC*), and bound to soluble streptavidin in order to orientate synopsis of DNA ends, was then added to YC beads and incubated with or without ATP. Consequently, only proteins bound to the DNA on beads in the first step were able to perform synopsis of DNA ends and the subsequent ligation reactions (66). Two washes were then performed to limit nonspecific association. YC fragments with a free blunt end (YC0) or with 2-, 4-, 6-, or 8-base complementary ends (YC2, YC4, YC6, and YC8, respectively) were used in parallel experiments. After the two-step pull-down reaction, soluble DNA was recovered by incubation of the DNA beads with AluI restriction enzyme, which allows us to differentiate the ligated YC duplex from the native unligated YC monomers. As shown in a representative experiment (Fig. 4A) and after quantification of the data (Fig. 4B), radiolabeled DNA was marginally retained on beads if NEs were omitted in the reaction. In contrast, with NE and in absence of ATP, a significant amount of YC* was

![Image](74x609 to 547x738)
trapped on beads whatever the extent of the 5’-overhang (Fig. 4A), leading to approximately the same quantity of radioactivity pulled down for all the oligonucleotides (Fig. 4B). Only unligated YC* monomers accounted for the pulled down radioactivity with 1 mM ATP, a ligation product with the expected length could be observed with ATP. Then two washes were performed to limit nonspecific association. For analysis of the pulled down DNA, beads were incubated with AluI endonuclease, and the digestion products were analyzed by electrophoresis on 20% polyacrylamide denaturing gels, and autoradiography of the gel was processed with a PhosphorImager (Storm System™, Amersham Biosciences).

End-joining Activity Is Independent of Classical NHEJ—The major DNA end-joining process in mammalian cells relies on the XRCC4/DNA ligase IV, Ku, and DNA-PKcs proteins (4, 66). Because we designed the pull-down assay in order to observe an end-joining activity independent of DNA-PK, we performed two lines of control experiments to verify this point. First, nuclear extracts were immunodepleted either for the Ku heterodimer or XRCC4 proteins by 20% polyacrylamide denaturing gels. As expected, the reaction was resistant to wortmannin, a DNA-PK inhibitor that covalently binds to the catalytic DNA-PKcs subunit. As expected, the reaction was resistant to wortmannin
shown is the mean of three experiments with S.D. DNA pressed as the percentage of ligated product out of total pulled down experiments with S.D. cation of total DNA pulled down as in amide denaturing gels after AluI digestion. pulled down DNA was analyzed by electrophoresis on 20% polyacryl-
experiments. The reaction was stopped at the indicated time, and the 3
protruding end (YC4 and YC4–3', respectively) were used in parallel
5
3
/YC4 and YC4/H11032
3
/H11541

very faint retention of radioactive YC4* DNA was detected
5
5

5
5

nucleotide. B, quantification of total DNA pulled down as in A. Shown is the mean of three experiments with S.D. C, quantification of the ligation efficiency, expressed as the percentage of ligated product out of total pulled down DNA. Squares, 5'-protruding termini; circles, 3'-protruding termini. Shown is the mean of three experiments with S.D.

(data not shown), contrary to DNA-PK-dependent end-joining in vitro (65).

The fact that we did not observe a DNA-PK-dependent NHEJ reaction under our conditions could be explained by the following: (i) the specificity of our two-step pull-down assay that would not allow synapsis in trans of two NHEJ proteins-DNA end complexes (66), and (ii) the preparation of NE used here, which differed from that of NHEJ-compliant extracts (65).

The End-joining Activity Is Dependent on PARP-1—We then investigated whether PARP-1 was involved in the end-joining activity detected in our assay. Under high concentration of β-NAD, poly(ADP-ribose) synthesis induces a loss of PARP-1 affinity for the DNA breaks (68). When 1 mM β-NAD was added to reaction buffer, only a marginal amount of radioactive YC4* DNA was pulled down and ligated (Fig. 7A, compare lanes 2 and 4), whereas α-NAD, which is not the natural PARP-1 substrate, had no effect on the reaction (Fig. 7A, compare lanes 2 and 3). Most interestingly, β-NAD also abolished the retention of radioactive YC4* DNA in the absence of ATP (Fig. 7A, compare lanes 5 and 6). These results indicate either that PARP-1 activity could modulate the synapsis and end-joining reactions that we observed or that its binding was necessary for these reactions to occur.

To distinguish between these two possibilities, we have tested NE from PARP-1−/− mouse embryonic fibroblasts. A very faint retention of radioactive YC4* DNA was detected with PARP-1−/− NE, and no end-joining occurred (Fig. 7B, lanes 5 and 6). However, addition of purified human recombinant PARP-1 protein to the reaction with PARP-1−/− NE restores both synapsis and formation of the YC4 joined product (Fig. 7B, lanes 7 and 8). Thus, PARP-1 was necessary for both synapsis and end-joining but could not account for the ligation activity. Therefore, we hypothesized that a ligase other than DNA ligase IV was involved in the reaction.

End-joining Activity Is Dependent on the XRCC1-DNA Ligase III Complex—PARP-1 interacts with XRCC1 (69), a protein also involved in BER (70), and XRCC1 is tightly associated with DNA ligase III (71). Because this protein complex could conceivably be responsible for the ligation step of the end-joining reaction observed here, we compared the activity of NE from XRCC1-deficient cells (EM9) (72) with NE from the parental line (AA8) in our YC4 DNA pull-down assay. As compared with AA8 extracts, we observed essentially no ligated product with EM9 NE (Fig. 7B, compare lanes 2 and 4) strongly suggesting that the XRCC1-associated ligase III was responsible for the ligation activity.

To assess the presence of PARP-1 and XRCC1 on the DNA beads, we performed Western blotting experiments on both the protein fractions retained on DNA beads and remaining in the supernatant (Fig. 7C). Without DNA on the beads, PARP-1 and XRCC1 remained in the supernatant (Fig. 7C, lane 1). In the presence of DNA, these two proteins associated with the DNA beads (Fig. 7C, lanes 2 and 3), but if β-NAD was added, less PARP-1 and XRCC1 remained in the DNA-bound protein fraction (Fig. 7C, lane 4). Note the mobility shift of PARP-1 associated with the beads, which corresponds to the poly(ADP)-ribosylated form of the protein (69), as assessed by reblotting the membrane with anti-PAR antibodies (data not shown).
With EM9 NE devoid of XRCC1, PARP-1 was still detected on the beads (Fig. 7C, lane 5), like with extracts from the AA8 parental line (Fig. 7C, lane 6). In contrast, no XRCC1 protein was retained on the DNA beads with NE extracts from PARP-1−/− cells (Fig. 7C, lane 7), showing that PARP-1 is necessary for XRCC1 recruitment on DNA, as already suggested (69). Indeed, addition of recombinant PARP-1 in NE extracts from PARP-1−/− cells promoted the recruitment of XRCC1 to the protein complex associated with the DNA beads (Fig. 7C, lane 8).

Because DNA ligase III is associated with XRCC1 (71), we assessed its presence by an adenylation assay on DNA beads preincubated with various NE (Fig. 8). A major adenylated protein was detected with HeLa NE with an apparent molecular mass of 105 kDa (Fig. 8, lane 1). The band intensity decreased when 5 pmol of YC4 was added after the reaction of ligase III (Fig. 8, lane 2). Moreover, adenylation was almost abolished when β-NAD was added to the NE (Fig. 8, lane 3), most likely due to dissociation of XRCC1 and PARP-1 from the DNA beads (see Fig. 7C, lane 4). When HeLa extracts immunodepleted for XRCC4, which contained only residual amounts of DNA ligase IV (Fig. 6A) (54), were used, an adenylation signal similar to the control undepleted HeLa NE was observed (Fig. 8, lane 4), indicating that DNA ligase IV did not correspond to the adenylated protein. We next tested NE from EM9 containing about 25% of DNA ligase III compared with extracts from the AA8 parental line (70). In contrast to AA8 NE that exhibited the same adenylated band as HeLa NE (Fig. 8, compare lanes 1 and 6), essentially no adenylated protein was detected with EM9 NE (Fig. 8, lane 5). These data allow to identify the major adenylated protein present on DNA beads as DNA ligase III and to establish that it is part of the synaptic complex.

Reconstitution of the End-joining Reaction with Recombinant Proteins—To confirm the results obtained with NE, we performed the YC4 end-joining reaction on DNA beads with only recombinant PARP-1 and the XRCC1-DNA ligase III complex (XL) (Fig. 9).

First, we tested the synapsis activity of XL or PARP-1. As shown in Fig. 9A, the presence of increasing amounts of XL did not promote retention of radiolabeled DNA above the amount that was marginally retained on beads in the absence of proteins in the reaction. In contrast, PARP-1 alone could promote DNA pull-down in a dose-dependent manner (Fig. 9A), and the presence of XL did not change significantly the synapsis activity of PARP-1. In the presence of ATP, this allowing ligation, XL alone promoted some end-joining in a concentration-dependent manner (Fig. 9B). However, addition of an optimal amount of PARP-1 for synapsis, as determined in Fig. 9A, strongly increased the yield of joined product (Fig. 9B, 7.5-, 6.2-, and 9.4-fold for 0.5, 1, and 2 molar ratios of XL to PARP-1, respectively). Addition of β-NAD strongly decreased both the amount of pulled down oligonucleotide and of ligated product. Taken together, these data confirm that both PARP-1 protein and the XL complex are necessary and sufficient in vitro for both the synapsis and ligation steps of the DSBs rejoining reaction under the experimental conditions reported here.

Finally, by using the reconstituted system with purified proteins, we tested the effect of the type of DNA ends on the end-joining reaction. As shown in Fig. 9C, the synapsis activity of PARP-1/XL was similar with 5′-, 3′-protruding or blunt DNA ends. Most interestingly, a noncomplementary 5′-protruding oligonucleotide was pulled down with a similar efficiency (Fig. 9C, lane 4). In contrast, the ligation efficiency was affected by the kind of DNA ends with a similar efficiency for 5′- or 3′-protruding ends, a lower efficiency for blunt ends, and no ligation with noncomplementary ends.
DISCUSSION

PARP-1 is an abundant nuclear protein that binds not only to SSBs but also to DSBs. Through its biochemical properties, PARP-1 is involved in DNA repair, recombination, and genomic stability (35–38). Although the affinity of PARP-1 for nicks is lower than for some types of DNA ends (34), most of the studies have concentrated on PARP-1 function in DNA repair via nick recognition (68).

We report here the involvement of PARP-1 in an end-joining reaction independent of the main DNA-PK reaction. Under cell-free reaction conditions, our results emphasize a new function of PARP-1 for synapsis of DNA ends that is uncoupled from the subsequent ligation step dependent on XRCC1/DNA ligase III (XL). We propose a model for a PARP-1-dependent DSBs-rejoining pathway (Fig. 10), the main features of which are the following. (i) Based on its high abundance and binding affinity for DSBs, it is likely that PARP-1 binds first to one end of the break, most probably as a PARP-1 catalytic homodimer (73); at this step and as shown here with the purified protein, PARP-1 is sufficient to bring DNA ends together. Then PARP-1 recruits XL to DNA ends. Although in our two-step pull-down assay, XL was recruited before the synapsis step, we cannot exclude that it could be recruited after this step. Anyhow, XL does not affect the synapsis activity of PARP-1. (ii) Finally, DNA ligase III reseals the break. Further characterization of the DNA-protein complexes would be necessary in order to know if two XL complexes associate with a PARP-1 homodimer.

This model is reminiscent of the reaction steps in the DNA-PK-dependent NHEJ mechanism in which DNA-PKcs recruited by Ku catalyzes DNA-ends synapsis (66), and then both end-positioned Ku and DNA-PKcs mediate the recruitment of the XRCC4-DNA ligase IV complex responsible for the ligation step (54).

By analogy with the current model for SSBs repair (52), XL recruitment may be favored by a low level of self-ADP-ribosylation of the enzyme (69). In vivo also, PARP-1 is necessary for H_{2}O_{2}-induced foci formation of XRCC1 that colocalize with sites of PAR synthesis (61, 74). Notably, both PARP-1 in nuclear extracts and as recombinant protein exhibited a reactivity on Western blots with anti-PAR antibodies specific for short PAR oligomers (data not shown). However, poly(ADP-ribosyl)ated PARP-1 has been shown to dissociate from DNA (68), and under these conditions, XL retained a high affinity for heavily automodified PARP-1 molecules (62). Indeed, we found that extensive modification impairs synapsis activity and promotes dissociation of both PARP-1 and XL from DNA. Thus, a small amount of PARP-1 self-ADP-ribosylation may allow XL recruitment onto DNA ends although subsequent extensive modification may promote dissociation of the protein-DNA complex. So, efficient end-joining is likely to require a tight coordination between the PARP-1 and DNA ligase activities at
DNA ends, probably resulting from a competition between the kinetics of both reactions.

Is this PARP-1-dependent DSBs rejoining activity in vitro relevant to a function in vivo? Here we have demonstrated a potentiation by a PARP-1 inhibitor of the cell sensitivity to the double-strand breaking agent calicheamicin γ1. In addition, we have reported a reduced kinetics of DSBs rejoining both in PARP-1-proficient cells pretreated with a PARP-1 inhibitor and in PARP-1-deficient cells, as assessed by loss of γ-H2AX phosphorylation. Because there was a clear potentiation of γ-H2AX phosphorylation in PARP-1−/− cells already detected after 1 h of drug treatment (Fig. 2), this observation is compatible with the possibility that a PARP-1-dependent DSB repair pathway operates early after DSB generation.

Altogether, these data support the possibility of the involvement of PARP-1 in vivo in an end-joining mechanism for DSBs, independent of DNA-PK. Accordingly, an impaired rejoining of DSBs repair after ionizing radiation has been demonstrated recently in PARP-1−/− cells, with only ~50% DNA DSBs rejoining within 60 min after irradiation (48). In addition, it was shown that a potentiation of ionizing radiation cytotoxicity by PARP-1 inhibition in both DNA-PK-proficient or -deficient rodent cells, which correlated with a severe and early inhibition of DNA DSBs rejoining as assessed by neutral filter elution (48, 50), is in full agreement with the sensitization effect by a PARP inhibitor both in DNA-PK-proficient or -deficient cells that we obtained here with calicheamicin γ1.

Although PARP-1 and XRCC1 have been implicated mostly in BER and single-strand breaks rejoining (75), several reports have already substantiated an involvement of these proteins in an end-joining pathway for DSB repair. XRCC1-deficient cell lines displayed a significant defect in rejoining of radiation-induced DNA DSBs (76, 77). In addition, XRCC1 is a key determinant of cell resistance to camptothecin, an indirect DNA-PKcs, inactivation of PARP-1 by genetic knock-out was inhibited a hyper-recombination phenotype (81). In cells lacking PARP-1-proficient cells pretreated with a PARP-1 inhibitor (45).

Regarding DSBs repair, PARP-1 was until now assigned to a sole anti-recombinogenic function. Its has been postulated that its binding to DNA might transiently prevent inappropriate recombination initiation (80). Accordingly, PARP-1−/− cells exhibited a hyper-recombination phenotype (81). In cells lacking DNA-PKcs, inactivation of PARP-1 by genetic knock-out was required for the occurrence of a low levels of VDJ recombination rescue (82), also arguing for a PARP-1 anti-recombinogenic function under these conditions. Actually, PARP-1 anti-recombinogenic activity could also rely in part on the synapsis function described here that might help to avoid the formation of free DSBs intermediates. Indeed, inactivation of both PARP-1 and Ku80 cellular activities by genetic knock-out in mice causes early embryonic lethality after probable improper attempts to repair extensive chromosome breaks due to endogenous DNA damage (83, 84).

The potential DSBs joining pathway involving enzymes of the BER pathway that we report here might be important if NHEJ becomes saturated or inefficient at a subset of DSBs. Indeed, substantial evidence in the literature argues for an alternative DNA-PK-independent end-joining pathway, based on both in vitro (18–23) and in vivo data (25–31). The fact that we found the same extent of potentiating effect of PARP-1 inhibition toward DSBs cytotoxicity in both DNA-PK-proficient and -deficient cells could be explained by a subclass of DNA DSBs that could solely be repaired by a PARP-1-dependent end-joining. If not repaired, these DNA breaks would impact on cell survival, despite an efficient DNA-PK end-joining. The additive potentiating effects of PARP-1 and DNA-PK inhibitors on both ionizing radiation cytotoxicity and inhibition of DNA DSBs rejoining (48) also support the hypothesis of independent end-joining mechanisms. Since it was reported recently that PARP-1 was not essential in vivo for repair of extrachromosomal I-SceI breaks by HR or NHEJ (85), we favor the involvement of PARP-1 in an alternative route of NHEJ dealing with damage that is a poor substrate of the DNA-PK machinery. For example, PARP-1 and XL-dependent DSB repair might realize a functional coupling between BER and end-joining; this pathway could operate on a subclass of DSBs arising from repair attempts at localized clusters of multiple base damage which are significantly induced by ionizing radiation (86, 87). In addition to genotoxic agents, stalled replication forks could also generate breaks that could be handled by a PARP-1-dependent repair pathway as suggested (85). This end-joining pathway could be responsible for the formation of chromosome aberrations observed in cells under NHEJ-deficiency conditions (30, 31, 86, 87). Because Ku-independent end-joining generally relies on microhomology-directed ligation (29, 32), our observation that PARP-1 can promote synthesis of noncomplementary DNA ends may suggest that it could be implicated in this pathway.

In conclusion, the data presented here help to elucidate the mammalian mechanisms responsible for the repair of DNA DSBs. They emphasized a potential role of PARP-1 as a key factor in an end-joining mechanism involved partly in cellular resistance to DSBs cytotoxicity. In addition, they suggest that PARP-1 could represent an important alternative cellular target in tumor radiosensitization strategy.

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Involvement of Poly(ADP-ribose) Polymerase-1 and XRCC1/DNA Ligase III in an Alternative Route for DNA Double-strand Breaks Rejoining
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