DNA-dependent Protein Kinase and XRCC4-DNA Ligase IV Mobilization in the Cell in Response to DNA Double Strand Breaks*

Jérôme Drouet‡§, Christine Delteil‡, Jacques Lefrançois‡, Patrick Concannon‡, Bernard Salles‡§, and Patrick Calsou‡

From the ‡Institut de Pharmacologie et de Biologie Structurale, CNRS UMR 5089, 205 route de Narbonne, 31077 Toulouse, Cedex 4, France and the §Benaroya Research Institute and the Department of Immunology, University of Washington, School of Medicine, Seattle, Washington 98101

Repair of DNA double strand breaks (DSBs) by the non-homologous end joining (NHEJ) pathway in mammals requires at least the DNA-dependent protein kinase (DNA-PK) and the DNA ligase IV-XRCC4 protein complexes. DNA-PK comprises the Ku70/Ku80 heterodimer and the catalytic subunit DNA-PKcs. Here we report the first description of the nuclear mobilization of endogenous NHEJ proteins after exposure of human cells to double strand-breaking agents. DSB infliction specifically induced a dose- and time-dependent mobilization of Ku70/80, DNA-PKcs, XRCC4, and DNA ligase IV proteins from a soluble nucleoplasmic compartment to a less extractable nuclear fraction. XRCC4 recruitment was accompanied by its DNA-PK-dependent phosphorylation. The recruited proteins co-immunoprecipitated, indicating that they had assembled into complexes. However, DNA-PK was attached to chromatin, whereas XRCC4-ligase IV resisted solubilization by DNase I. The rates of appearance and dissolution of NHEJ proteins paralleled that of histone variant H2AX phosphorylation and dephosphorylation. We established that under conditions of genomic DSB infliction 1) Ku recruitment was not dependent on the co-recruitment of the other NHEJ proteins, 2) DNA-PKcs was physically required for the mobilization of the XRCC4-ligase IV complex, 3) DNA ligase IV was physically necessary for stable recruitment of XRCC4, and 4) phosphorylation of either H2AX or XRCC4 was unnecessary for DNA-PK or XRCC4-ligase IV recruitment. Altogether these results offer insights into the interplay between key NHEJ proteins during this repair process in the cell.

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

In eukaryotic cells, DSBs are repaired through two distinct pathways: homologous recombination and non-homologous end joining (NHEJ) (3–5). NHEJ, the major pathway in mammalian cells, requires several factors that recognize and bind the DSBs, catalyze the synopsis of the broken ends, and then process and reseal the break (6, 7).

Major insights into the mechanism of NHEJ have been gained from in vitro assays with cell-free extracts or purified native or recombinant components. NHEJ repair of a DNA break is initiated by loading of the DNA-dependent protein kinase (DNA-PK). DNA-PK consists of a regulatory subunit, the Ku70/Ku80 heterodimer, that recognizes and binds to DNA ends and a catalytic subunit (DNA-PKcs or p460) that exhibits serine-threonine kinase activity (8, 9). The determination of the structure of Ku70/80 bound to a DNA end has shown that it forms an asymmetric ring around the DNA helix (10). Also p460 has an end bridging activity (11). The XRCC4-DNA ligase IV complex is responsible for the break resealing step (12, 13).

We have established that both DNA-PK subunits are required for the recruitment of the XRCC4-DNA ligase IV complex to model DNA breaks (14). Also the Artemis protein has a role at least in the resolution of hairpin-ended breaks (15, 16), and polynucleotide kinase removes a 5'-phosphate group or adds a missing phosphate at 5'-ends (17). Additional factors may also be involved in the NHEJ pathway (18, 19). The protein kinase activity of DNA-PKcs is necessary per se for functional end joining based on several criteria. (i) Phosphorylation is required for activation of key substrates as in the case of the cryptic endonuclease activity of Artemis responsible for hairpin opening (15). (ii) Autophosphorylation of p460 is required for its dissociation from Ku (20), for DSB repair (21–23), and most probably for deprotection of DNA ends to make them accessible to subsequent gap-filling or ligation steps (24, 25).

Proteins involved in various DNA repair pathways have been shown to relocalize in response to DNA damage. However, very few studies have addressed the question of the nuclear mobilization of NHEJ factors in vivo following DSB induction most likely due to the background resulting from high levels of NHEJ factors in mammalian cells. Chan et al. (21) reported no change in the strong nuclear overall staining for p460 in x-ray-irradiated cells, although a specific anti-phosphorylated Thr-2609 revealed a subpopulation of discrete foci after exposure to IR. Photobleaching experiments using HeLa cells expressing Ku70- or Ku80-green fluorescent protein fusion constructs showed that Ku moves rapidly throughout the nucleus even 30

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‡ To whom correspondence should be addressed. Tel.: 33-5-61-17-59-36; Fax: 33-5-61-17-59-33; E-mail: bernard.salles@ipsbs.fr.

† The abbreviations used are: DSB, DNA double strand break; NHEJ, non-homologous end joining; DNA-PK, DNA-dependent protein kinase; DNA-PKcs or p460, catalytic subunit of DNA-PK; Ncs, neocarzinostatin; IR, ionizing radiation; PBS, phosphate-buffered saline; RT, room temperature; WCE, whole cell extract; ATR, ataxia-telangiectasia-muta-ted and Rad3-related; ATM, ataxia-telangiectasia-mutated.
Mobilization of NHEJ Proteins in Vivo

min following IR (26). However, the functional repair activity of these constructs was not assessed in this study, preventing extrapolation of these conclusions to the endogenous Ku protein pool.

Here we analyzed the nuclear mobilization in human cells of the major DNA-PK and XRCC4-ligase IV NHEJ proteins in response to DSB at early time points following damage infliction. We describe a novel detergent-based cellular fractionation protocol that allowed us to assess in situ the DSB-induced recruitment of untagged endogenous NHEJ repair proteins as visualized by both immunofluorescence and immunoblot analyses. Altogether our findings represent the first description in the cell of the nuclear mobilization and interplay of key NHEJ proteins after exposure to double-strand-breaking agents.

MATERIALS AND METHODS

Chemicals—Neocarzinostatin was a kind gift from Dr. V. Favaudon (Institut Curie, Orsay, France). It was stored as a 1 mM stock solution in 10 mM sodium citrate buffer, pH 4.0 at −80 °C and diluted in the same buffer. Bleomycin was purchased from Sigma. Cisplatin (cis-diaminedichloroplatinum II) was a gift from Roger Bellon Cie. Bleomycin (10 mM stock solution) and cisplatin (3 mM stock solution) were dissolved in water and 150 mM NaCl, respectively, and stored at −20 °C. Wortmannin (Sigma) was dissolved in Me2SO (10 mM stock solution) and stored at −20 °C. Small aliquots of stock solution chemicals were used once.

Antibodies—Anti-Ku70 (N3H10), anti-Ku80 (clone 111), anti-Ku70/80 (clone 162), and anti-p460 (DNA-PKcs, clone 15.2) monoclonal antibodies were from Neomarkers (Fremont, CA). Monoclonal antibody anti-phosphorylated H2AX (JXB301) and anti-lamin A/C (clone 636) were from Upstate Cell Signaling Solutions and Santa Cruz Biotechnology, respectively. Polyclonal rabbit antibodies anti-XRCC4 and anti-ligase IV were from Serotec Ltd. (Oxford, UK) and from Santa Cruz Biotechnology, UK, respectively. The polyclonal rabbit antibody anti-Rad51 and the monoclonal antibody anti-HP1α (clone 2H9-2G9) were gifts from Dr. M. Defais (Institut de Pharmacologie et de Biologie Structurale, Toulouse, France) and Dr. R. Losson (Institut de Génétique et de Biologie Moleculaire et Cellulaire, Illkirch, France), respectively. Peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Culture and DNA-damaging Treatments—All culture media were from Invitrogen and were supplemented with 10% fetal calf serum unless indicated, 2 mM glutamine, 125 units/ml penicillin, and 125 µg/ml streptomycin. HCT116 cells (a gift from Dr. F. Praz, Institut Gustave-Roussy, Villejuif, France) were maintained in Dulbecco’s modified Eagle’s medium. MRC5-SV cells (a gift from Dr. A. Sarasin, Institut Gustave-Roussy, Villejuif, France) were maintained in Dulbecco’s modified Eagle’s medium. The lymphoblastoid cell lines (GM8920, Coriell Institute, Camden, NJ, and LB2304 (27) were grown in RPMI 1640 medium with 15% fetal bovine serum and 1 mM sodium pyruvate. DNA-PKcs-deficient and -complemented cell lines (Fu9S, alias M059J, and Fu1S, respectively (28), gifts from Dr. C. Kirchgesner, Stanford University School of Medicine) were maintained in Dulbecco’s modified Eagle’s medium (F-12:11.1 medium. All cells were grown in a humidified atmosphere at 37 °C with 5% CO2. Before drug exposure, exponentially growing cells were washed with unsupplemented medium either in culture dishes or by centrifugation for cell suspensions that were either mock-treated or treated with chemicals at the specified concentrations in unsupplemented medium at 37 °C in culture dishes or in 15-mL tubes for lymphoblastotyping and centrifuged. For cell suspensions, cells were collected by centrifugation and washed as above. For Biochemical Fractionation and Immunoblotting—Treated or mock-treated cells in culture dishes were washed twice with ice-cold PBS, cells were scraped anti-radiating detergent, and centrifuged. For cell suspensions, cells were collected by centrifugation and washed as above. Cell fractionation was carried out by two consecutive extractions. The supernatant was collected at each step and labeled as fractions S. Pellets of about 2 × 10⁸ cells were first resuspended for 15 min on ice in 200 µl of extraction buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 0.1% Triton X-100 supplemented with protease inhibitor mixture tablets (Complete Mini™, Roche Diagnostics) and phosphatase inhibitors (10 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1 mM cantharidin, all from Sigma). Following centrifugation at 14,000 × g for 3 min, the supernatant was collected, and the pellet was further resuspended for 15 min on ice in 200 µl of extraction buffer. The extracts were clarified by centrifugation at 14,000 × g for 3 min, and the supernatant was pooled with the previous one (fraction S1). The pellet was further incubated in 200 µl of extraction buffer without Triton but supplemented with 200 µg/ml RNase A (Sigma) for 30 min at 25 °C under agitation. Following centrifugation at 14,000 × g for 3 min, the supernatant (fraction S2) was separated from the pellet (fraction P2). When necessary, the P2 pellet was incubated for 1 h at 37 °C in the presence of 100 units of calf intestine phosphatase (New England Biolabs) in 20 mM Tris-HCl, pH 8, 2 mM magnesium chloride, and protease inhibitors as above. When necessary, a third extraction was performed for 30 min at 37 °C by resuspension of the P2 pellet in extraction buffer without detergent supplemented with 5 mM manganese chloride and 300 µg/ml DNase I (Sigma). Following centrifugation at 14,000 × g for 3 min, the supernatant (fraction S3) was separated from the pellet (fraction P3). Insoluble P2 or P3 fractions were resuspended in PBS buffer supplemented with 1% SDS, heated for 10 min at 100 °C, and sonicated for 10 s (Vibracell, Bioblock Scientific). Whole cell extracts of treated or mock-treated cells were obtained by direct lysis in PBS buffer supplemented with 1% SDS and treatment as above. Concentrated protein buffer was used for a 1× final concentration in all fractions, and the samples were boiled for 5 min. Equal aliquots of each fraction, derived from equivalent cell numbers, were separated by SDS-PAGE (8% for standard separation or 15% for γ-H2AX isolation) and blotted onto polyvinyldiene difluoride membranes (Hybond-P, Agram, Biosciences). Membranes were blocked for 1 h in 5% dry milk in PBS containing 0.1% Tween 20 (PBS-T) and incubated for 1 h with primary antibody diluted in PBS containing 0.02%. Tween 20 and 1% bovine serum albumin (fraction V, Sigma). After three washes with PBS-T, membranes were incubated for 1 h with secondary antibodies in PBS containing 0.02% Tween 20 and 5% dry milk. Immunoblots were visualized by enhanced chemiluminescence (Super Signal, Pierce). Providing extensive washing and probing first with polyclonal antibodies, successive immunoblotting were performed on the same membranes for extended periods. For dual-color staining, films were scanned and processed with Adobe Photoshop 3.0 software.

Immunoprecipitations—For Ku70/80 immunoprecipitations, anti-Ku70/80 (162) antibodies were coupled to magnetic anti-mouse IgG beads (Dynabeads M-450, Dynal), and for XRCC4 immunoprecipitations, anti-XRCC4 antibodies were coupled to magnetic anti-rabbit IgG beads (Dynabeads M-280, Dynal) according to the manufacturer’s protocol. P2 pellets of untagged NHEJ proteins were treated or mock-treated with antibodies against Ku70/80, and treated or mock-treated HCT116 cells were sonicated for 10 s in 200 µl of extraction buffer. Then under 100-µl final buffer volume, 30 µl of extracts were incubated at 4 °C for 180 min under gentle agitation with 10 µl of beads in 25 mM Hepes-KOH (pH 7.5), 100 mM NaCl, 20% glycerol, 5 mM EDTA, 5 mM di-thiothreitol, 0.1% Nonidet P-40, 10 mM NaF, 10 mM sodium orthovanadate, 1 mM cantharidin (Sigma), and protease inhibitor mixture tablets (Complete Mini, Roche Diagnostics). The supernatant was removed over a magnet (Dynal MPC, Dynal). After two washes in the same buffer, proteins in the immunoprecipitates were heated in SDS sample buffer.

In Situ Detergent Extraction and Immunofluorescence—Cells grown for 48 h on glass coverslips to about 70% confluence in 24-wells plates were untreated or treated with CsA as described above. After 1 h, cell extraction was carried out in situ by incubating the coverslips in extraction buffer as described above for 2 min at room temperature (RT). Control cells were treated in the same buffer without detergent. Cells were fixed in 3.7% paraformaldehyde for 20 min at RT followed by a 5-min incubation with 0.2% Triton X-100 in PBS at RT. After each step, conjugated-antibodies were rinsed three times with PBS. Coverslips were blocked with 10% fetal calf serum, 2% bovine serum albumin, 0.2% Triton, and 100 µg/ml RNase A in PBS for 60 min at RT. Then cells were incubated with the appropriate primary antibody (1:300 dilution in PBS, 2% fetal calf serum, 0.2% Triton) for 1 h at RT. After extensive washing with PBS, 2% fetal calf serum, antibody binding was detected by the application of a 1:300 dilution of fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson, PA), goat anti-mouse IgG (Jackson), and rhodamine red-conjugated goat anti-rabbit IgG (both from Molecular Probes) for 30 min at room temperature. The DNA was stained with either a 1:500 dilution of TO-PRO-3 iodide (Molecular Probes) for 30 min at room temperature or 2 µg/ml propidium iodide (Sigma) for 5 min at RT. Coverslips were mounted in Vectorshield (Vector Laboratories). Confocal images were obtained by means of a confocal laser microscopy.
Fig. 1. Immunostaining of NHEJ proteins in untreated and neocarzinostatin-treated HCT116 cells. A, HCT116 cells grown on coverslips were mock-treated or treated with 1 μM neocarzinostatin (+ Ncs) for 1 h at 37 °C in unsupplemented medium and then fixed, permeabilized, and immunostained with primary antibodies against Ku70/80 heterodimer (a and e) or XRCC4 protein (c and g) followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse or rabbit IgG, respectively. DNA was stained with propidium iodide (b, d, f, and h). B, HCT116 cells grown on coverslips were treated or not with 1 μM neocarzinostatin (+ Ncs) for 1 h at 37 °C in unsupplemented medium and then permeabilized in buffer containing 0.1% Triton X-100, fixed, and immunostained with primary antibodies against Ku70/80 heterodimer (a and g), XRCC4 protein (c and i), or lamin A/C (e and h) followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse or rabbit IgG as required. DNA was stained with propidium iodide (b, d, f, h, j, and l).

RESULTS

Nuclear Retention of NHEJ Proteins after Treatment of Cells with a DNA Double Strand Break-inducing Agent—We reasoned that gentle extraction of the nucleoplasmic pool of the key NHEJ proteins could allow the visualization by immunofluorescence of a potential nuclear association of these proteins at early time points following DSB induction. This approach has been used to observe the retention of other repair proteins (29, 30), but it has not been applied previously to NHEJ proteins. Thus, to remove loosely bound nuclear proteins, we adapted a cellular fractionation procedure based on detergent extraction (30). HCT116 colon carcinoma cells were grown on glass coverslips and either treated or not with the radiomimetic drug Ncs for 1 h. Ncs is a natural enediyne antibiotic that has been shown to produce DSBs with selectivity and efficiency higher than IR (31) and to efficiently induce DSBs and cytotoxicity when applied to cells (32). To optimize DSB production and thus the potential recruitment of repair proteins, Ncs was used at a dose about 10-fold higher than the IC50, determined on theses cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test after 48 h of culture (data not shown). After a 1-h drug treatment, cells were either extracted or not with Triton, then fixed, and immunostained for proteins representative of the early damage recognition and later rescaling steps of the NHEJ pathway (Ku70/80 heterodimer and XRCC4, respectively). An antibody against lamin A/C was used as a control for nuclear scaffold proteins (33). In non-extracted cells, Ku and XRCC4 showed a diffuse nuclear staining with the exception of nucleoli or mitotic chromosomes that were not stained. No change in the nuclear staining pattern of both proteins was detected upon treatment with Ncs (Fig. 1A). After the in situ cellular extraction procedure with Triton, images obtained by phase-contrast clearly showed that only the nuclei remained (data not shown). Nuclear staining of both Ku and XRCC4 proteins was lost in untreated cells, whereas nuclear lamin A/C was still readily detectable (Fig. 1B, left panels). In contrast, Ku and XRCC4 were retained in nuclei of Ncs-treated cells under the same detergent extraction conditions (Fig. 1B, right panels). Identical results were obtained with DNA-PKcs and DNA ligase IV staining or after treatment with the other enediyne antibiotic γ-calicheamicin (data not shown). Thus, the removal of nucleoplasmic or loosely bound NHEJ proteins enabled clear detection of retained proteins in the nuclei of cells treated with DSB-inducing molecules within a 1-h incubation time.

Isolation of Protein Fractions Resistant to Detergent Extraction after Treatment of Cells with DSB-inducing Agents—To analyze biochemically the nuclear protein fraction retained after detergent extraction following DSB induction, we adapted the protocol used above to immunostain cells scraped from culture dishes. HCT116 colon carcinoma cells were extracted with a 0.1% Triton-containing buffer, and the clarified cell extract was collected (S1). The cell pellet was treated with RNase A in the same buffer but without detergent, and the soluble and insoluble fractions were collected (S2 and P2, re-
and blotted with anti-XRCC4 antibodies. With calf intestine phosphatase (CIP), analyzed by SDS-PAGE as in A, and blotted with anti-XRCC4 antibodies. A parallel extraction procedure was performed on untreated cells and cells treated with Ncs. The membranes were blotted with the antibodies as indicated.

**FIG. 2.** Analysis of protein fractionation in untreated and neocarzinostatin-treated HCT116 cells and effect of calf intestine phosphatase treatment. HCT116 cells in culture dishes were treated or not with 1 μM neocarzinostatin (+Ncs) as described in Fig. 1. Cells were collected and lysed in denaturing buffer (WCE) or fractionated by two consecutive extractions as described under “Materials and Methods,” leading to S1 and S2 soluble fractions and P2 insoluble material. A, protein samples were denatured and separated by SDS-PAGE (8% for standard separation or 15% for γ-H2AX and Rad51 isolation) followed by electrotransfer on membrane. The membranes were blotted with the antibodies as indicated. B, P2 insoluble material was treated with calf intestine phosphatase (CIP), analyzed by SDS-PAGE as in A, and blotted with anti-XRCC4 antibodies.

respectively). A parallel extraction procedure was performed on untreated cells and cells treated with Ncs. Fig. 2A shows the immunoblot analysis following SDS-PAGE of cell-equivalent aliquots of the three fractions compared with whole cell extracts (WCEs) under both untreated and Ncs-treated conditions. The membranes were blotted with antibodies against Ku70, Ku80, XRCC4, DNA ligase IV, and γ-H2AX, the latter serving as a quantitative nuclear marker of DSB (34). In addition, anti-Rad51 antibodies were used as a probe of the homologous recombination route for DSB repair. As opposed to non-treated cells, WCEs from Ncs-treated cells contain γ-H2AX as expected given the high DNA DSB inducing potency of Ncs (32). In addition, XRCC4 in the WCEs of Ncs-treated cells was detected essentially as a slow migrating form. In untreated cells, the majority of the proteins detected were released during the first (XRCC4-DNA ligase IV and Rad51) or the two extraction steps (Ku), and none of these proteins were detected in the insoluble P2 fraction. In contrast, both the S2 and/or P2 fractions from Ncs-treated cells were highly enriched in NHEJ proteins, including the slowly migrating form of XRCC4. In addition, γ-H2AX was exclusively present in the insoluble P2 fraction from Ncs-treated cells, whereas no Rad51 protein was retained under these conditions.

The presence of DSB in cells has been shown to induce phosphorylation of XRCC4, retarding its migration in SDS-PAGE (35). Accordingly we found that the XRCC4 slower migrating form was sensitive to calf intestine phosphatase, indicating that it corresponded to a phosphorylated form (Fig. 2B).

We then analyzed the specificity of protein recruitment toward the class of DNA lesions. NHEJ-defective mutants are selectively sensitive to DSB-inducing agents (36), and the Ku dimer exhibits a strong binding activity toward DSBs with no sequence specificity (37). Accordingly we found a selective retention of NHEJ proteins (including the p460 DNA-PK catalytic subunit) in the P2 insoluble fraction following treatment of cells with the DSB-inducing agents Ncs and bleomycin (Fig. 3A). In addition, a similar recruitment was observed in response to cell treatment with γ-calicheamicin and high doses of ionizing radiation (data not shown). There was no significant retention of NHEJ proteins when these cells were heavily irradiated with UV-C rays or treated with high doses of the cross-linking agent cisplatin or the DNA methylating molecule methyl methanesulfonate (MMS, 1 mM, bleomycin (Bleo, 140 μM), and Ncs (1 μM). After 1-h incubation time in unsupplemented medium, cells were collected and lysed in denaturing buffer (WCE) or fractionated by two consecutive extractions as described under “Materials and Methods,” leading to P2 insoluble material. Protein samples were denatured and separated by SDS-PAGE followed by electrotransfer on membrane and blotting with the antibodies as indicated. B, HCT116 and MRC5-SV cells were treated or not with 140 μM bleomycin, incubated, collected, extracted, and separated as in A. The membranes were blotted with the antibodies as indicated.

**FIG. 3.** Effect of the class of DNA-damaging agent and of the cell type on the recruitment of NHEJ proteins in a nuclear insoluble fraction. A, HCT116 cells in culture dishes were treated or not with UVC light (UV1 and UV2; 80 and 240 J/m², respectively), cis-diamminedichloroplatinum II (CisPl1 and CisPl2; 60 and 180 μM, respectively), methyl methanesulfonate (MMS, 1 mM), bleomycin (Bleo, 140 μM), and Ncs (1 μM). After 1-h incubation time in unsupplemented medium, cells were collected and lysed in denaturing buffer (WCE) or fractionated by two consecutive extractions as described under “Materials and Methods,” leading to P2 insoluble material. Protein samples were denatured and separated by SDS-PAGE followed by electrotransfer on membrane and blotting with the antibodies as indicated. The membranes were blotted with the antibodies as indicated.

Since HCT116 cells have been reported to be defective in the mismatch repair MLH1 (38) and to exhibit a low expression of the MRE11 protein (39), both traits that could potentially modulate the NHEJ pathway, we checked NHEJ repair protein recruitment in another cellular background. As shown in Fig. 3B, MRC5 lung fibroblastic cells treated with bleomycin also exhibited selective retention of Ku and p460 DNA-PK components together with XRCC4 and DNA ligase IV in the P2 insoluble fraction when compared with the untreated cells. Again Rad51 was not retained in the non-extractable fraction under these conditions.

These observations indicate that at short times after selective induction of DSBs in cells, a subset of DNA-PK and
XRCC4-DNA ligase IV complexes, but not the key homologous recombination protein Rad51, become resistant to detergent extraction, indicating a tighter association with nuclear components. In addition, depending on the DSB-inducing agent, XRCC4 could be retained in a normal (with bleomycin) or a slow migrating form (with Ncs).

**NHEJ Proteins Are Recruited as a Complex, and XRCC4-Ligase IV Specifically Interacts with a DNase I-resistant Nuclear Compartment**—We then asked whether NHEJ proteins were assembled as a DSB-induced complex. We performed co-immunoprecipitation experiments on the P2 fraction from Ncs-treated or untreated cells in parallel with antibodies against XRCC4. As shown in Fig. 4A, NHEJ proteins were only recovered from the P2 fraction of Ncs-treated cells. Notably, the XRCC4-ligase IV complex co-immunoprecipitated with DNA-PKcs using antibodies directed against Ku, and conversely the DNA-PK complex was immunoprecipitated together with DNA ligase IV using antibodies against XRCC4. As a control, γ-tubulin did not co-precipitate with Ku or XRCC4 (data not shown).

To determine whether the NHEJ proteins in the detergent-insoluble fraction were bound to chromatin, we examined the effect of DNA digestion. The Ncs-treated cells were extracted as before (soluble fraction S1/2), and the insoluble fraction P2 was treated with DNase I, leading to an S3 supernatant and a P3 pellet. As shown in Fig. 4B, DNA digestion released γ-H2AX as expected for a chromatin component but also Ku, which became almost undetectable in the DNA-resistant P3 fraction. Other experiments showed that DNA-PKcs was also mostly solubilized by DNA digestion (data not shown). As a control, the nuclear scaffold protein lamin A/C was absent in the S3 protein fraction solubilized by DNase while still abundant in the P3 pellet. In contrast to Ku, the XRCC4-ligase IV recruited complex was resistant to the DNase treatment and was found together with lamin A/C in the P3 fraction (Fig. 4B). Taken together, these data suggest that, in the presence of DSBs, NHEJ proteins assemble into complexes in which DNA-PK binds to chromatin, whereas XRCC4-ligaseIV interacts with DNase I-resistant nuclear structures.

**Dose Dependence and Time Course of NHEJ Protein Retention**—We then examined the time course of protein retention in the extraction-resistant fraction P2 in HCT116 cells after exposure to Ncs. Fig. 5A shows that γ-H2AX formation was detected at 5 min, the earliest time point examined. It has been reported that γ-H2AX foci are formed at sites of DSBs as early as 1 min after damage infliction (40). Fig. 5A shows that the kinetics of NHEJ protein retention correlates well with the appearance of γ-H2AX. In addition, XRCC4 showed a retention pattern with the first appearance of an intermediate migrating form at 5 min followed by progressive accumulation of an even slower migrating form, most likely corresponding to a multiple phosphorylated form. A similar stepwise phosphorylation process has already been reported during phosphorylation of other DNA repair proteins, such as replication protein A following UV irradiation (41). Interestingly DNA ligase IV accumulated in the retained protein fraction in parallel to XRCC4 independently of XRCC4 phosphorylation status. As a control of protein loading, we used the HP1α protein, which is very strongly attached to chromatin (42).

We next tested the dose dependence of protein retention by detergent extraction of HCT116 cells following exposure to increasing concentrations of Ncs. As already found (Fig. 2), the retention of NHEJ proteins is correlated with the appearance of γ-H2AX (Fig. 5B). Under these conditions, NHEJ proteins and γ-H2AX were detectable at a Ncs dose of 0.1 μM in the P2 fraction and accumulated proportionally with higher doses. Again an intermediate form of XRCC4 was retained at the lowest Ncs dose and then evolved to a more slowly migrating form at higher doses (Fig. 5B).

We also examined the dose dependence of protein retention in the fraction P2 in HCT116 cells after exposure to bleomycin (Fig. 5C). Again a clear dose response was observed for the retention of NHEJ proteins under these conditions, correlated with the appearance of γ-H2AX. Interestingly comparison between extracts from bleomycin- and Ncs-treated cells showed a strong difference in the phosphorylation status of the recruited XRCC4 fraction; although both radiomimetic chemicals induced γ-H2AX expression and similar recruitment of the NHEJ proteins, phosphorylation of XRCC4 recruited by bleomycin was barely detected and only for high doses.

To follow the kinetics of dissociation of the retained NHEJ proteins, HCT116 cells were exposed to Ncs for 10 or 40 min and then incubated in a drug-free medium for 2 or 4 h. As shown in Fig. 5D, NHEJ proteins were detected for a longer time in the detergent-resistant fraction for the longest time exposure to the radiomimetic chemical (compare time point 2 h for 10 and 40 min of drug exposure). However, nuclear retention of both Ku and XRCC4 decreased after 4 h postincubation in close correlation with the disappearance of γ-H2AX. In addition, loss of XRCC4 retention coincided with loss of its phos-
phorylation form as shown in the S1 fraction after detergent extraction (Fig. 5C, bottom blot).

These results indicate that the doses of DNA-damaging compounds used to visualize the mobilization of the NHEJ proteins remain below the repair capacity of the cells, which can remove most of the DSBs as shown by γ-H2AX disappearance. Accordingly efficient rejoining of Ncs-induced DSBs was observed using neutral elution for the detection of DSBs (32) or disappearance of γ-H2AX foci (30). Since γ-H2AX is also produced during apoptosis (43), the loss of γ-H2AX that we observed also indicates that cells are not undergoing apoptotic DNA fragmentation at least during the time course of the experiment. Finally these observations also suggest that retention of NHEJ proteins and γ-H2AX formation occur concomitantly after DSB production and then decrease at the same rate following damage repair.

NHEJ Protein Retention Is Not Inhibited by Wortmannin—
The antifungal agent wortmannin is a potent inhibitor of the three different phosphatidylinositol 3-kinase-like kinases, DNA-PK, ATM, and ATR (44). Thus, it was used to investigate whether such a DNA damage-activated kinase activity was required for the damage-induced nuclear retention of NHEJ proteins. It was tested in parallel in the two cellular backgrounds, HCT116 and MRC5. As shown in Fig. 6, wortmannin strongly decreased (HCT116) or abolished (MRC5) XRCC4 damage-induced phosphorylation in the detergent-resistant protein fraction from Ncs-treated cells. However, unphosphorylated XRCC4 was still retained in both cases, indicating that XRCC4 phosphorylation was

FIG. 5. Time course and dose response of the recruitment and release of NHEJ proteins in response to neocarzinostatin or bleomycin (Bleo). HCT116 cells were treated or not with the indicated chemical. Then cells were collected and lysed in denaturing buffer (WCE) or fractionated by two consecutive extractions as in Fig. 2 leading to P2 insoluble material. Protein samples were denatured and separated by SDS-PAGE (8% for standard separation or 15% for γ-H2AX and HP1α isolation) followed by electrotransfer on membrane and blotting with the antibodies as indicated. A, cells were treated with 1 μM neocarzinostatin for the incubation time as indicated. B, cells were treated with the indicated concentration of neocarzinostatin for 1 h. C, cells were treated or not with the indicated concentration of bleomycin for 1 h. D, cells were treated with 1 μM neocarzinostatin in unsupplemented medium for the indicated incubation time, then washed, and incubated in complete medium without the drug for the indicated recovery period. The XRCC4 content is compared between the insoluble P2 and pooled S1 + S2 (S1/2) protein fractions.

FIG. 6. Effect of wortmannin (Wortm) on the recruitment and release of NHEJ proteins in response to neocarzinostatin. HCT116 or MRC5 cells were preincubated for 2 h in the absence or in the presence of the indicated concentration of wortmannin in complete medium and then treated or not with 1 μM neocarzinostatin for 1 h in the presence of the same concentration of wortmannin. Then cells were collected and lysed in denaturing buffer (WCE) or fractionated by two consecutive extractions as in Fig. 2 leading to P2 insoluble material. Protein samples were denatured and separated by SDS-PAGE (8% for standard separation or 15% for γ-H2AX) followed by electrotransfer on membrane and blotting with the antibodies as indicated.
not necessary for its DSB-induced nuclear retention. DNA ligase IV and Ku were also still efficiently recruited to the detergent-resistant fraction in the presence of wortmannin. ATM and DNA-PK are required for the optimal phosphorylation of H2AX after exposure to DSB agents (45). As expected, H2AX phosphorylation was strongly inhibited (HCT116 cells) or abolished (MRC5 cells) in the presence of wortmannin (Fig. 6) as previously reported (40). Since NHEJ proteins were still recruited under this condition, this indicates that γ-H2AX is not required for their DSB-dependent mobilization.

DNA Ligase IV Is Required for an Optimal XRCC4 Recruitment—By monitoring protein assembly in vitro on DNA ends, we have reported that the recruitment of the XRCC4-DNA ligase IV complex was strictly dependent on the assembly of both the Ku and p460 components of DNA-PK to these ends (14). In addition, data suggested that DNA ligase IV was physically required for an interaction with DNA-PK at DNA breaks (46, 47). Using the assays described above, we tested this hypothesis by assessing the DSB-induced assembly of NHEJ proteins in the lymphoblastoid cell line LB2304 derived from a patient bearing two truncating mutations in the LIG4 genes (27). LB2304 expresses a very low level of residual truncated protein undetectable by Western blotting or immunoprecipitation (27), whereas the XRCC4 protein level is normal (Ref. 48 and Fig. 7). A control lymphoblastoid cell line was treated and fractionated by two consecutive extractions as in Fig. 2 leading to P2 insoluble material. Protein samples were denatured and separated by SDS-PAGE followed by electrotransfer on membrane and blotting with the antibodies as indicated. WT, wild type.

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Taken together, these data establish that DNA-PKcs is dispensable for the DSB-induced recruitment of Ku but physically necessary for that of XRCC4-ligase IV.

**DISCUSSION**

Most of the current model for the NHEJ repair mechanism of DNA DSBs has been gained from experiments *in vitro* with cell extracts or purified components mixed with linear plasmids or oligonucleotides. In contrast, we present here insights into the interplay of the key DNA-PK and XRCC4-ligase IV NHEJ proteins in the cell in response to DSBs and establish some steps for their assembly *in vivo* during the repair mechanism.

First, we report that in untreated cells, most of Ku70/80, XRCC4, and DNA ligase IV proteins are in the soluble nucleoplasmic compartment since we observed that in the absence of DSB (i) the nuclear immunostaining of these proteins was lost after detergent extraction and (ii) these proteins were exclusively found in the soluble protein fractions as shown by biochemical analysis. Only one study has previously addressed this point and found by fluorescence photobleaching that green fluorescent protein fusion constructs of either Ku70 or Ku86 showed a rapid movement of the entire pool of Ku proteins throughout the nucleus (26).

Second, we have shown that DSBs induce the mobilization of NHEJ proteins to a detergent-resistant nuclear compartment as visualized by both immunofluorescence and Western blot. These proteins comprise at least DNA-PKcs, Ku70/80, XRCC4, and DNA ligase IV. The association of these proteins in the nucleus is dose- and time-dependent, and this protein mobilization is specifically initiated by DSB-inducing agents. Early time points following DSB induction were chosen to avoid potential interference with later processes like apoptosis or homologous recombination. Indeed we did not detect cell apoptosis within the 1-h drug treatment used here (data not shown) nor did we observe any DSB-induced recruitment of the key homologous recombination protein Rad51 (Figs. 2 and 3B). Importantly these results reflect the *in situ* assembly of endogenous functional NHEJ repair proteins and avoid potential bias due to bulky tags. Accordingly we have not observed under the same extraction conditions a DSB-induced recruitment with either an N-terminal green fluorescent protein-tagged Ku70 or an N-terminal cyan fluorescent protein-tagged XRCC4 fusion protein expressed in human cells.  

What is the relation between the NHEJ recruitment that we observed and the DSB repair process *per se*? This recruitment shows a clear dependence on the specific generation of DSBs and is unlikely to represent any general cellular response to DNA damage. The time course of appearance of this recruitment paralleled that of the DSB-specific induction of H2AX phosphorylation. In addition and for the milder doses of Ncs, the disruption of NHEJ protein recruitment and dephosphorylation of γ-H2AX occurred concomitantly with a kinetics compatible with the rejoicing kinetics of Ncs-induced DSBs generated in normal cells (32). The mobilized NHEJ proteins associate in complexes as demonstrated by their co-immunoprecipitation from the P2 fraction of drug-treated cells (Fig. 4A). In addition, we observed that most of the DNA-PK recruited fraction was released upon treatment with DNase I indicating that this assembly takes place on chromatin. Thus, it is most likely that the mobilization of NHEJ enzymes to a less extractable nuclear compartment that we observed is a *bona fide* reflection of their assembly onto sites of DNA DSBs and conversely that the reversion of the recruitment represents the disassembly of the repair machinery from the repaired

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2 J. Drouet, C. Delteil, P. Frit, P. Concannon, B. Salles, and P. Calsou, unpublished results.
breaks. Interestingly purification of the protein complexes recruited under our conditions could allow the identification of new partners of the core repair proteins.

We have shown that NHEJ complex assembly still occurred under conditions where H2AX phosphorylation was impaired (Fig. 6). These data clearly establish that γ-H2AX formation is not necessary for NHEJ recruitment in the presence of DSBs in vivo, consistent with the fact that H2AX is not required for NHEJ (49).

We demonstrated that DSB-induced Ku mobilization can occur in the absence of DNA-PKcs or DNA-ligase IV. This strongly argues that the binding of Ku to the DNA ends represents the first limiting step of the NHEJ process as suggested in vitro (for a review, see Ref. 37). Interestingly Ku recruitment was amplified in the physical absence of its catalytic partner at a low level of DSBs (Fig. 8 and data not shown). This agrees with our observations in vitro that one role of the kinase subunit is to limit the spreading of Ku onto the DNA molecule (24, 50).

Our data clearly show that DNA-PKcs is physically required for recruiting XRCC4-ligase IV. This directly substantiates the suggestions that DNA-PK could target the ligase activity to sites of DNA damage (14, 47, 51, 52). Our present data may be partly explained by the direct interaction reported between DNA-PKcs and XRCC4 (51). In vitro, Ku alone stimulates DNA ligase IV-dependent ligation by recruitment of the XRCC4-ligase IV complex, providing Ku could move inward on the DNA (46). Interestingly DNA-PKcs causes inward translocation of Ku (24, 53). Thus, another function of DNA-PKcs with regard to XRCC4-ligase IV recruitment may be to provide accessibility to DNA ends. DNA-PKcs might be necessary for most of the DSBs, although the repair of a minor subclass of breaks could escape this requirement. For example the rejoining of signal junctions during VDJ recombination occurs almost normally in the absence of DNA-PKcs (54). This could explain the minor DSB-induced XRCC4-ligase IV recruitment that persists in the DNA-PKcs-deficient Fus9 cells (Fig. 8).

We established that DNA ligase IV is physically required for optimal recruitment of XRCC4-ligase IV to the NHEJ repair complex. This recruitment could rely partly upon a direct interaction between Ku and ligase IV (47). Experiments with purified components have shown that an intact adenylation site in ligase IV is required (46). Although the DSB-induced association of XRCC4 with the P2 fraction of Ncs-treated ligase IV mutant cells was not detected under our standard extraction conditions, XRCC4 was fully phosphorylated in the WCEs of these cells (Fig. 7). This could indicate that a transient and unstable recruitment of XRCC4 may take place even in the absence of ligase IV, possibly relying on a direct contact between XRCC4 and DNA-PKcs (51). Nevertheless our results indicate that other interactions involving DNA ligase IV are necessary to stabilize this assembly. Interestingly we found that in the recruited NHEJ complexes, DNA-PK binds to chromatin, whereas XRCC4-ligase IV interacts with DNase I-resistant nuclear structures (Fig. 4B). Experiments with cell extracts have shown that XRCC4-ligase IV is part of a protein complex preferentially associated with nuclear matrix attachment DNA sequences (55). The C terminus of ligase IV has two BRCA1 C-terminal domains shown to be phosphoserine- or phosphothreonine-binding modules (56). One possibility is that these motifs may be involved in the interaction between the XRCC4-ligase IV complex and an unknown target in the nuclear matrix, the phosphorylation of which would be induced upon DSB detection. Further experiments are needed to address this possibility.

In the absence of DNA ligase IV, we report also that the DSB-induced mobilization of DNA-PK is normal (Fig. 7). It can be predicted from this result that if normal ligation is impaired, DNA-PK binding at sites of DSBs hinders subsequent handling of the damage by alternative pathways such as homologous recombination. This would explain the observation that Ku inactivation can rescue some defects of ligase IV-deficient cells (57–59) and the embryonic lethality of ligase IV-deficient mice (60).

In Ncs-treated cells, we clearly observed a time- and dose-dependent recruitment of XRCC4 associated with its phosphorylation. Matsumoto et al. (35) have shown that an IR-induced phosphorylation of XRCC4 was abolished in the DNA-PKcs-deficient M059J cells. However, this cell line also exhibits a strong defect in ATM expression (61) that may also possibly impair this phosphorylation. Here the restoration of the break-induced XRCC4 phosphorylation in the DNA-PKcs-deficient cells allows us to conclude unambiguously that DNA-PKcs is the main kinase responsible for DSB-induced phosphorylation of XRCC4. In addition, although DNA-PK-dependent phosphorylation of XRCC4 abrogates its binding to DNA in vitro (62), our present data indicate that in the cell, phosphorylated XRCC4 is also present in the protein fraction recruited by broken genomic DNA.

The role of XRCC4 phosphorylation in the cell is still a subject of debate (63, 64). In Ncs-treated cells in the presence of wortmannin or in cells treated with bleomycin, sub- or unphosphorylated XRCC4 was found in the nuclear insoluble fraction. This indicates that XRCC4 phosphorylation by DNA-PK is dispensable for its DSB-induced recruitment and suggests that phosphorylation occurs after XRCC4 recruitment. From our data, we can also infer that XRCC4 phosphorylation is unlikely to regulate interaction with DNA ligase IV since we found that both proteins were equally co-recruited under permissive or preventive phosphorylation conditions. Accordingly a deletion mutant of XRCC4 lacking the region encompassing the phosphorylation sites has been shown to still complement repair deficiency (52, 62). Interestingly most of the cellular pool of XRCC4 was phosphorylated under the highest doses of Ncs used so that the soluble protein fraction also contained fully phosphorylated XRCC4 (Fig. 2). Also phosphorylation of this soluble XRCC4 reverted at the same rate as γ-H2AX (Fig. 5D) suggesting that it is linked to completion of the DSB end joining. Thus, soluble phosphorylated XRCC4 per se could serve a function, like for example, signalling the ongoing repair process to other cellular machineries.

Comparison of the time course and dose-response profiles for XRCC4 recruitment between extracts from bleomycin- and Ncs-treated cells showed a strong difference in the phosphorylation status of the recruited XRCC4 fraction, whereas other NHEJ proteins were recruited similarly. Although both radiomimetic chemicals induced γ-H2AX expression indicative of their production of DSBs, the XRCC4 recruited fraction after treatment with bleomycin was poorly phosphorylated at late time points or high doses, whereas the recruited XRCC4 observed with Ncs treatment was predominantly phosphorylated even at early time points or low doses. This could indicate that XRCC4 phosphorylation may require a threshold level of a specific subclass of lesions that may be reached by different doses for each of these radiomimetic drugs. This possibility is currently under investigation and could shed light on a function of XRCC4 phosphorylation for the repair of some special breaks.

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