Repair of DNA double strand breaks (DSB) by the nonhomologous end-joining pathway in mammals requires at least seven proteins involved in a simplified two-step process: (i) recognition and synopsis of the DNA ends dependent on the DNA-dependent protein kinase (DNA-PK) formed by the Ku70/Ku80 heterodimer and the catalytic subunit DNA-PKcs in association with Artemis; (ii) ligation dependent on the DNA ligase IV-XRCC4-Cernunnos-XLF complex. The Artemis protein exhibits exonuclease and endonuclease activities that are believed to be involved in the processing of a subclass of DSB. Here, we have analyzed the interactions of Artemis and nonhomologous end-joining pathway proteins both in a context of human nuclear cell extracts and in cells. DSB-inducing agents specifically elicit the mobilization of Artemis to damaged DNA and is the main kinase that phosphorylates Artemis in cells damaged with highly efficient DSB producers. Under kinase preventive conditions, both in vitro and in cells, Ku-mediated assembly of DNA-PK on DNA ends is responsible for a dissociation of the DNA-PKcs-Artemis complex. Conversely, DNA-PKcs kinase activity prevents Artemis dissociation from the DNA-PK-DNA-PKcs complex. Altogether, our data allow us to propose a model in which a DNA-PKcs-mediated phosphorylation is necessary both to activate Artemis endonuclease activity and to maintain its association with the DNA end site. This tight functional coupling between the activation of both DNA-PKcs and Artemis may avoid improper processing of DNA.

DNA double strand breaks (DSB) in cells are produced by exogenous damaging agents like ionizing radiation (IR) or radiomimetic molecules but also endogenously as by-products of oxidative metabolism or perturbation of the DNA replication fork. In addition, tissue-specific DSB are produced during specialized processes like meiosis in germinal cells or V(D)J recombination in lymphocytes. Improper signaling or repair of DSB in cells can lead to cell death or cancer-prone genomic rearrangements (1, 2).

DSB are mainly repaired through two distinct pathways: homologous recombination and nonhomologous end joining (NHEJ), but DSB are mainly processed by the latter pathway in mammalian cells. NHEJ requires several factors that recognize and bind the DSB, catalyze the synopsis of the broken ends, and then process and reseal the break (3–5). Although alternative subpathways for NHEJ may operate in cells (6, 7), the major pathway relies on a set of core proteins, the individual deficiency of which elicits both IR sensitivity and V(D)J recombination defect (2, 8). In humans or animals, these defects are responsible for a radiosensitive severe combined immunodeficiency (RS-scid) syndrome (9).

The DSB is recognized and bound by the asymmetric ring-shaped heterodimer Ku70/Ku80 that recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (10). The assembled DNA-PK holoenzyme then exhibits serine-threonine protein kinase and DNA end-bridging activities (reviewed in Refs. 11 and 12). One of the kinase functions is to regulate DNA end access to processing enzymes by means of an autophosphorylation operation (13–15). The XRCC4-DNA ligase IV complex is responsible for the ligation step (16, 17), and both Ku and DNA-PKcs components are necessary to load this complex to the site of the break (18, 19). Recently, a new core NHEJ factor with structural similarity to XRCC4, Cernunnos-XLF, has been identified concomitantly as deficient in a human RS-scid syndrome (20) and as an XRCC4-interacting protein (21). The exact role of this factor is still unknown, but it has been postulated to function in all NHEJ events based on its interaction with XRCC4 (21), the high IR sensitivity of the corresponding deficient cells (20, 21), and their complete defect in NHEJ activity in vitro (20, 22).

Another NHEJ factor is Artemis, which was originally identified, like Cernunnos, as deficient in a human RS-scid syndrome (23). Cells derived from these patients show an increased sensitivity to IR (24–26). Gene targeting in mice broadly reproduced these findings (27, 28). Artemis exhibits an intrinsic single strand-specific 5’ to 3’ exonuclease activity but has also hairpin opening activity in vitro mediated by its DNA-PKcs-dependent phosphorylation (29). This corroborates the...
findings that a lack of Artemis in vivo leads to V(D)J recombination defects analogous to that produced by a DNA-PKcs deficiency (i.e., impaired coding joining in human cells (26, 30) and unresolved coding end hairpins in animals (28)). In addition, DNA-PKcs activates a versatile endonuclease activity of Artemis that can cleave various substrates near the single- to double-stranded transition region (29, 31), and the Artemis catalytic core for V(D)J recombination has been mapped (32, 33). Although the initial kinetics of DSB repair is normal in Artemis cells (25), Artemis has been proposed to be responsible for the processing of some kinds of IR-generated DNA DSB, since deficient cells have a subtle defect in late repair of DSB (34, 35). DNA-PKcs-dependent phosphorylation sites in the C-terminal portion of the Artemis have been suggested to have an important regulatory role in the activity of the protein (31), most of them not being SQ or TQ consensus DNA-PKcs sites (31). In addition, an ATM- and ATR-dependent phosphorylation of Artemis has been reported in cells (34, 36, 37, 38), but its relation to Artemis function in DSB repair is not yet fully understood. Moreover, Artemis could have another role in the regulation of cell cycle progression following DNA damage, including UV irradiation (35, 38), although this cell cycle function was not confirmed by others (39). Nevertheless, Artemis clearly has a caretaker function, since fibroblasts from Artemis-deficient mice show genomic instability (27, 28), and Artemis-deficient patients as well as Artemis/p53-deficient mice show chromosomal instability and show a predisposition to lymphomas (41).

Although cellular studies have established a role for Artemis in the repair of minor DSB and biochemical experiments have documented its nuclease activity, this protein is still the NHEJ factor for which the least is known about its interactions with the other components of the reaction. It has been reported to form a complex with and to be phosphorylated by DNA-PKcs, leading to the activation of its endonuclease activity (29). However, under these experimental conditions with purified components, DNA-PKcs was activated without Ku, and no role was observed for Ku in the overhang processing or the hairpin opening by the Artemis-DNA-PKcs complex (29, 42). This implies that important aspects of the interactions between Artemis and the other core NHEJ components may have been overlooked. With this in view, we have analyzed the interactions of Artemis and NHEJ proteins on DNA ends by incubating human nuclear cell extracts with paramagnetic beads bearing double-stranded oligonucleotides and characterizing proteins bound to DNA ends. We next have challenged and validated in cells our in vitro results by using a detergent-based cellular fractionation protocol that allows us to assess in situ the DSB-induced recruitment NHEJ repair proteins (19).

**MATERIALS AND METHODS**

**Oligonucleotide Substrates**—Oligodeoxyribonucleotides were purchased from Sigma-Genosys (France). The blunt-end C0 double-stranded DNA fragment was constructed by annealing the 32-nucleotide oligomer 5′-TAAAGGGACAAACAGCTGGGTACCGGTGTTCG-3′ biotinylated on the 5′-end with the complementary nonbiotinylated oligonucleotide. The 5′ Cn protruding oligonucleotides were constructed by annealing the 32-mer biotinylated as above with complementary nonbiotinylated oligonucleotides bearing various protruding 5′ ends (5′-CGCGG-3′ for C2, 5′-CGATCGG-3′ for C4, and 5′-CGTTA-ACG-3′ for C6, respectively).

**Chemicals**—Calicheamicin γ1 (Cal), a generous gift from P. R. Hamann (Wyeth Research, Pearl River, NY), was dissolved in ethanol and stored at −70 °C. Nocarzinostatin was a kind gift from Dr. V. Favaudon (Institut Curie, Orsay, France). It was stored as 1 mM stock solution in 10 mM sodium citrate buffer, pH 4.0, at −80 °C and diluted in the same buffer before use. Cisplatin (cis-diaminedichloroplatinum-II) was a gift from Roger Bellen Cie. Cisplatin (3 mM stock solution) was dissolved in 150 mM NaCl and stored at −20 °C. Wortmannin (Sigma) and NU7026 (Calbiochem) were 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), anti-p460 (DNA-PKcs, clone 18.2), and anti-actin (clone ACTN50) monoclonal antibodies were from Neomarkers. Monoclonal antibody anti-c-Myc (clone 9E10), anti-phosphorylated H2AX (JBW301), and anti-lamin A/C (clone 636) were from BD Biosciences, Upstate Cell Signaling Solutions, and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Polyclonal rabbit antibody anti-XRCC4 and anti-ligase IV were from Serotec and from Abcam, respectively. The polyclonal rabbit antibody anti-Rad51 and the monoclonal antibody anti-HP1α (clone 2HP-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 Moléculaire et Cellulaire, Illkirch, France), respectively. Peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories.

**Cell Culture and Extracts**—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. Guetel are SV40T-transformed, telomerase-immortalized Artemis-deficient fibroblasts, and Guetel-A were obtained after transduction of Guetel with pMND-Artemis-Myc-ires-GFP retroviral vector expressing a C-terminal Myc-His-tagged Artemis protein (37); both cell lines were grown in RPMI medium. DNA-PKcs-deficient and complemented cell lines (Fus9, alias M059J, and Fus1, respectively (43) (gifts from Dr. C. Kirchgessner, Stanford University School of Medicine) were maintained in Dulbecco’s modified Eagle’s medium/F-12 1:1 medium. All cells were grown in a humidified atmosphere, at 37 °C with 5% CO2. Nuclear protein extracts were prepared as previously described (18), except that the final dialysis was performed for 3 h at 4 °C in an excess volume of dialysis buffer as follows: 50 mM Tris-HCl, pH 7.5, 10% glycerol, 100 mM potassium glutamate, 1 mM EDTA, 1 mM dithiothreitol. After preparation, all of the extracts were immediately frozen and stored at −80 °C.

**Peptide and Purified Protein**—The recombinant N-terminal hexa-His-Ku70/Ku80 heterodimer was expressed in SF9 cells using baculovirus-based expression vector as previously described (44) and purified on a Hitrap chelating column (Amersham Biosciences) charged with Ni2+ ions followed by
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HitrapQ HP (Amersham Biosciences) anion exchange chromatography. The C80 peptide (GSGSEGDDVDLDMDI) was synthesized by H. Mazarguil (Institut de Pharmacologie et de Biologie Structurale) and corresponds to the last 12 C-terminal amino acids of Ku80 with a GSGS linker to a biotin moiety at the N terminus as published (45). The peptide buffer used as control was 25 mM Tris-HCl, pH 8.8.

Ku Immunodepletions and Artemis Immunoprecipitations—For Ku immunodepletions, anti-Ku70/80 (162) antibodies were coupled to magnetic anti-mouse IgG beads (Dynabeads M-450; Dynal) according to the manufacturer’s recommendations. Then 250 µg of nuclear protein extracts were incubated at 4 °C for 60 min under gentle agitation with 20 µl of beads in dialysis buffer. The supernatant was removed over a magnet (Dynal MPC; Dynal). A second depletion was performed immediately under the same conditions. For Artemis immunoprecipitation, anti-Myc antibodies were added to Guetel-A nuclear extracts in IP buffer (25 mM Hepes-KOH (pH 7.5), 100 mM NaCl, 20% glycerol, 5 mM EDTA, 1 mM dithiothreitol, 0.05% Nonidet P-40, 10 mM NaF, 0.2 mM sodium orthovanadate, 1 mM cantharidin (Sigma), and protease inhibitor mixture tablets as recommended by the manufacturer (Roche Applied Science) and incubated for 2 h at 4 °C under agitation, and then 20 µl of protein A-immunobeads (Dynal) were added per reaction and further incubated for 90 min at 4 °C under agitation. Then the beads were washed with IP buffer and used as necessary.

DNA End Binding Assay—Five pmol of double-stranded oligonucleotide as indicated were immobilized on 10 µl of streptavidin paramagnetic beads (Dynabeads M280 streptavidin; Dynal) in 50 µl of 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM NaCl for 30 min at 20 °C under agitation. After washes with the same buffer, DNA- or mock-treated beads were incubated in 25 µl of reaction mixture containing 30 µg of nuclear extracts in standard reaction buffer (40 mM Hepes-KOH (pH 7.8), 5 mM MgCl2, 60 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 3.4% glycerol, 0.3 mg/ml bovine serum albumin) and 1 mM ATP when necessary. For conditions without ATP, extracts were first incubated for 10 min at 30 °C in standard reaction buffer without ATP, supplemented with 2 mM glucose and 0.2 units of hexokinase (Sigma) in order to remove traces of ATP as described (18). For conditions with Wortmannin, extracts were preincubated in the presence of 30 µM Wortmannin in reaction buffer without oligonucleotide and ATP for 10 min at 30 °C. Control extracts were similarly preincubated in parallel. Incubation was for 30 min at 30 °C under agitation. The extract supernatant was removed, and the beads were mixed gently on a wheel for 3 h at 4 °C. For anti-Artemis IPs, anti-Myc antibodies were first added to the reaction mixture and incubated for 2 h at 4 °C under agitation, and then 20 µl of protein A-immunobeads (Dynal) were added per reaction and further incubated for 90 min at 4 °C under agitation. The beads were pulled down over a magnet, the extract supernatant was removed, the beads were washed twice with 1 ml of IP buffer, and proteins in the immunoprecipitates were heated in SDS sample buffer and separated in a 15% acrylamide Tris-glycine-SDS gel.

DNA-damaging Treatments and Transfection—Before drug exposure, exponentially growing cells were washed with unsupplemented medium, either mock-treated or treated with chemicals, at the specified concentrations in unsupplemented medium at 37 °C in culture dishes and then harvested at the indicated time points. For UV irradiation, cells were washed with phosphate-buffered saline (PBS) and then exposed to UVC irradiation (254 nm) with a germicidal lamp (Bioblock). Immediately after irradiation, unsupplemented medium was added, and cells were postincubated as above. Fus9 and Fus1 cells were transiently transfected with the Nucleofector II apparatus (Amatox) as follows: 2 × 106 cells were transfected with 3 µg of pcDNA1.1-Artemis-myc vector (37) in 100 µl of Nucleofector V buffer (program A23, about 43% transfection efficiency) and then diluted 15-fold and incubated for 48 h in complete medium.

Biochemical Fractionation and Immunoblotting—Treated or mock-treated cells in culture dishes were washed twice with ice-cold PBS, collected by scraping, and centrifuged. Cell fractionation was carried out by two consecutive extractions. The supernatant was collected at each step and labeled as fraction S. Pellets of about 2 × 106 cells were first resuspended in 3 ml 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 Applied Science) 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 (fraction S1), and the pellet was washed with extraction buffer without Triton. The pellet was further incubated in 100 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, which was then washed with extraction buffer without Triton (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. Insoluble P2 fraction was resuspended in PBS buffer supplemented with 1% SDS, heated 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 loading sample buffer was added for 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 on SDS-PAGE (8% for standard separation or 15% for γ-H2AX and HP1α isolation) and blotted onto...
polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp.). 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 (ImmunofaxA; Yelen). After extensive washing and probing first with polyclonal antibodies, successive immunoblots were performed on the same membranes without stripping. For data presentation, films were scanned and processed with Adobe Photoshop 3.0 software.

RESULTS

DNA-PK Kinase Activity Is Necessary to Maintain the DNA-PKcs/Artemis Association during Binding to DNA Ends in Vitro—First, we have focused on the protein fraction bound to DNA ends in vitro in order to evaluate the capacity of Artemis to be recruited to DNA ends in the context of nuclear extracts. Nuclear extracts from the Guel-A fibroblasts expressing a C-terminal Myc-tagged Artemis protein easily detected on Western blot were incubated with paramagnetic streptavidin beads bearing a double-stranded oligonucleotide modified with a biotin moiety at one 5′ end as target DNA. Proteins bound to DNA ends were analyzed by Western blotting. In addition, to assess the role of DNA-PKcs in the assembly of repair proteins onto DNA ends, the reaction was performed either with ATP or in the presence of the known DNA-PK inhibitor wortmannin.

As shown in Fig. 1, DNA-PKcs, Ku70/80, and Myc-tagged Artemis were present in the protein fraction retained on DNA with no nonspecific binding to the paramagnetic beads in the absence of DNA. Ku80 bound to DNA was shifted to a slightly slower form under ATP conditions corresponding to phosphorylated Ku80 as reported (18). A quantitative modulation was observed for DNA-associated Artemis and DNA-PKcs; DNA-PKcs accumulated on the DNA beads in the presence of wortmannin. When necessary, Guetel-A nuclear extracts were preincubated in the presence of wortmannin (+ wortmannin) or an enzymatic ATP-depleting system (− ATP), before contacting paramagnetic beads bound or not with double-stranded oligonucleotides carrying various 5′ extensions (0, 2, 4, and 6 nucleotides for C0, C2, C4, and C6, respectively) in standard reaction buffer supplemented or not with ATP. After incubation for 30 min at 30°C, the beads were washed and heated in SDS sample buffer, and then the bound protein fractions were separated in 8% SDS-PAGE and analyzed by Western blotting with antibodies as indicated. Artemis was detected with an anti-Myc antibody. The arrows show electrophoretic mobility shifts.

FIGURE 1. Binding of Artemis and DNA-PK on DNA beads and the effect of wortmannin. When necessary, Guetel-A nuclear extracts were preincubated in the presence of wortmannin (+ wortmannin) or an enzymatic ATP-depleting system (− ATP), before contacting paramagnetic beads bound or not with double-stranded oligonucleotides carrying various 5′ extensions (0, 2, 4, and 6 nucleotides for C0, C2, C4, and C6, respectively) in standard reaction buffer supplemented or not with ATP. After incubation for 30 min at 30°C, the beads were washed and heated in SDS sample buffer, and then the bound protein fractions were separated in 8% SDS-PAGE and analyzed by Western blotting with antibodies as indicated. Artemis was detected with an anti-Myc antibody. The arrows show electrophoretic mobility shifts.

Another explanation for the data of Fig. 1 was that Ku/DNA-PKcs interaction on DNA ends destabilized the DNA-PKcs/Artemis complex under kinase-preventive conditions. In order to analyze the composition of the protein complex assembled on DNA ends under kinase permissive or preventive conditions, we performed protein assembly on free double-stranded oligonucleotides followed by immunoprecipitation experiments with either anti-DNA-PKcs or anti-Ku antibodies and checked for coimmunoprecipitation with Artemis (Fig. 2). None of the three proteins were precipitated by the anti-actin control antibody (Fig. 2, lanes 6 and 11). In contrast, the anti-polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp.). 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 (ImmunofaxA; Yelen). After extensive washing and probing first with polyclonal antibodies, successive immunoblots were performed on the same membranes without stripping. For data presentation, films were scanned and processed with Adobe Photoshop 3.0 software.

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DNA-PKcs and anti-Ku antibodies precipitated very efficiently their respective target from the nuclear extracts (Fig. 2, compare lanes 2 and 7 with lane 1). In the absence of added DNA, Ku and DNA-PKcs marginally co-precipitated (Fig. 2, lanes 2 and 7) as expected (43); in contrast, Ku and DNA-PKcs co-precipitated in the presence of free double-stranded oligonucleotides under kinase-preventive conditions (Fig. 2, lanes 3 and 8, without ATP, and lanes 5 and 10, with wortmannin), corresponding to the DNA-PK complex stalled at DNA-ends. On the contrary and as expected, ATP dissociated the DNA-PK complex (Fig. 2, lanes 4 and 9). Artemis co-precipitated with DNA-PKcs but not Ku in nuclear extracts in the absence of DNA (Fig. 2, compare lanes 2 and 7), as already reported (29); note that only a fraction of the protein is engaged in a complex with DNA-PKcs in extracts from Guetel-A cells. Under conditions in which a whole DNA-PK-DNA complex was formed, Artemis remained associated with the complex only when DNA-PKcs was active, and it co-precipitated as a phosphorylated form (Fig. 2, lanes 4 and 9). These data confirmed our hypothesis that the Artemis-DNA-PKcs complex was stabilized when DNA-PK assembled on DNA unless the kinase was active.

Ku Is Responsible for DNA-PKcs/Artemis Dissociation upon DNA-PK Binding to DNA Ends in Vitro—In order to strengthen these conclusions, we performed a similar immunoprecipitation experiment with standard and Ku-immunodepleted nuclear extracts in parallel and analyzed the DNA-PK components co-immunoprecipitating with Artemis (Fig. 3). The anti-Myc antibody precipitated efficiently Artemis from the nuclear extracts (Fig. 3, compare lane 2 with lane 1). Only a fraction of DNA-PKcs associated stably with Artemis (Fig. 3, lane 2). In control extracts, we found again that Artemis remained associated with DNA-PKcs only when the latter was active (+ ATP), and under these conditions, it co-precipitated as a phosphorylated form, together with Ku and DNA-PKcs (Fig. 3, top, compare lane 4 with lanes 3 and 5). In contrast, no significant variation was detected in the amount of DNA-PKcs co-precipitated with Artemis from the Ku-depleted extracts, whatever the incubation conditions (Fig. 3B, bottom). Low salt conditions promote a Ku-independent DNA-PKcs binding to DNA (47) and Artemis phosphorylation with ATP (29). Thus, we have checked the stability of the DNA-PKcs-Artemis complex on anti-Myc immunomembranes incubated with DNA under such conditions, in the presence of Ku and in the presence or not of ATP. No significant release of DNA-PKcs from the beads was detected, whatever the incubation conditions (data not shown). Taken together, these results indicate that the Ku-mediated assembly of DNA-PK on DNA ends was responsible for a dissociation of the DNA-PKcs-Artemis complex under kinase-preventive conditions.

In order to focus on DNA-PK assembly onto DNA, we reconstituted the reaction with purified fractions (Fig. 4). The DNA-PKcs-Artemis complex was first immunoprecipitated from nuclear extracts without DNA and then incubated with double-stranded oligonucleotides in the presence or absence of various concentrations of purified Ku heterodimer (Fig. 4A). DNA plus Ku conditions promoted a dose-dependent loss of DNA-PKcs from the Artemis immunoprecipitates, whereas neither DNA nor Ku alone had any effect on DNA-PKcs/Artemis association. The extreme C-terminal domain of Ku80 has been shown to be sufficient for association with DNA-PKcs in the absence of DNA (45, 48). Thus, we mimicked the Ku/DNA-PKcs interaction by using a C80 peptide derived from Ku80 C terminus (45). When the DNA-PKcs-Artemis complex was immunoprecipitated as above, the addition of the C80 peptide was sufficient to promote DNA-PKcs/Artemis dissociation (Fig. 4B, compare lanes 1 and 2). In contrast, when the DNA-PKcs/Artemis was immunoprecipitated under conditions promoting DNA-PK assembly and Artemis phosphorylation (ATP plus DNA (Fig. 4B, note the shift of Artemis and the presence of Ku in lanes 3 and 4)), the Ku80 C-terminal peptide instead dissociated Ku from the complex, and most of DNA-PKcs still precipitated with phosphorylated Artemis (Fig. 4B, compare lanes 3 and 4).

Taken together, these data from in vitro experiments establish that Ku binding to DNA-PKcs in the presence of double-stranded DNA, most probably via its extreme C terminus, promotes Artemis dissociation from DNA-PKcs unless the kinase is active.

Artemis Is Recruited to Chromatin Containing DSB in Cells—In order to validate our previous conclusions in cells, experiments were then performed in Guetel-A fibroblasts expressing the Myc-tagged Artemis construct after stable retroviral transduction of Gunetel-Artemis-deficient cells (37). Guetel-A fibroblasts were either treated or not with drugs producing DSB formation, Cal and neocarzinostatin (Ncs). Cal and Ncs are natural enediyne antibiotics that have been shown to produce DSB with selectivity and efficiency higher than IR (49) and to efficiently induce DSB and cytotoxicity when applied to
cells (50, 51). Fibroblasts established from Artemis-defective patients as well as Artemis−/− mouse embryo fibroblasts from Artemis−/− mice show increased sensitivity to IR (26–28) as well as to the radiomimetic drug bleomycin (27). Accordingly, we observed also a marked increased sensitivity of Guetel fibroblasts to the radiomimetic drugs Cal and Ncs, which was fully restored in Guetel-A cells by expression of Myc-tagged Artemis (data not shown).

We have described recently a detergent-based cellular fractionation protocol allowing us to assess in situ the DSB-induced recruitment of the main NHEJ repair proteins, as visualized by immunoblot analysis (19). This protocol was applied to Guetel-A cells in order to check for Artemis mobilization to chromatin after DSB infliction. Since Cal yields a 1:3 ratio of DNA DSB to single-stranded breaks in vivo, compared with a 1:20 ratio for IR (50), we have chosen this radiomimetic drug to treat the cells. After 1 h of drug treatment, cell nuclei were extracted with a Triton-containing buffer, and the clarified cell extract supernatant was collected (S1) after centrifugation. The cell pellet was treated with RNase A in the same buffer but without detergent as described (19), and the soluble and insoluble fractions were collected after centrifugation (S2 and P2, respectively). A parallel extraction procedure was performed on untreated and damaged cells after Cal treatment. Fig. 5A shows the immunoblot analysis following SDS-PAGE of cell-equivalent aliquots of the three fractions compared with whole cell extracts (WCE), under both untreated and Cal-treated conditions. Proteins were detected by antibodies against Artemis, DNA-PKcs, Ku80, DNA ligase IV, XRCC4, and γH2AX, the phosphorylated form on serine residue 139 of the histone H2AX variant, which is admitted to be a quantitative nuclear marker of DSB (52). In addition, an anti-Rad51 antibody was used to probe the homologous recombination (HR) route for DSB repair. WCE of Artemis-deficient cells serve as a negative control for Artemis expression (Fig. 5A, lane 1). As opposed to nontreated cells, WCE from Cal-treated cells contain γH2AX (Fig. 5A, lane 2), in agreement with the high DNA double-stranded breaking potency of Cal. In untreated cells, the majority of NHEJ proteins was released during the two extraction steps, and only a marginal amount was detected in the insoluble P2 fraction, whereas on the contrary, the P2 fraction from Cal-treated cells was highly enriched for these proteins, including Artemis (Fig. 5A, compare lanes 8 and 9). Also, γH2AX was exclusively present in the insoluble P2 fraction from Cal-treated cells. In contrast, Rad51 protein was detected identically in the P2 fraction of drug-treated and nontreated cells. In addition, Artemis and XRCC4 in all of the fractions of Cal-treated cells were detected essentially as slowly migrating forms that were sensitive to calf intestine phosphatase (data not shown), corresponding to phosphorylated forms, as already reported for XRCC4 (19).

Since Artemis-defective mutants are selectively sensitive to DSB-inducing agents, we then analyzed the specificity of protein recruitment toward the class of DNA lesions (Fig. 5B). As expected for the recruitment of a key NHEJ protein, we found that phosphorylated XRCC4 was retained in the P2 insoluble fraction following treatment of cells with the DSB-inducing agents Ncs and Cal, in correlation with the appearance of γH2AX. Notably, the retention of Artemis paralleled that of XRCC4, and the recruitment of Artemis was accompanied by its phosphorylation. In addition, Artemis was similarly recruited after cell treatment with bleomycin and IR (data not shown). In contrast and when compared with the untreated cells, there was no significant retention of both Artemis and XRCC4 proteins when these cells were heavily irradiated with UV-C rays or treated with the cross-linking agent cisplatin (Fig. 5B). Then the time course of protein retention in the extraction-resistant fraction P2 in Guetel-A cells after exposure to Cal was examined. Fig. 5C shows that γH2AX formation was detected at 5 min, the earliest time point examined, and that the kinetics of Artemis, Ku, and XRCC4 protein retention was in close synchrony with the appearance of γH2AX, as already shown for the core NHEJ proteins (19). In addition, Artemis and XRCC4 showed a similar retention pattern with the appearance of an intermediate migrating form, followed by progressive accumulation of an even slower migrating form, most likely corresponding to multiple phosphorylated forms.
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The kinetics of Cal-induced Artemis phosphorylation is in agreement with the one reported after IR (35, 37).

**DNA-PKcs Is Necessary for the Recruitment of Artemis to DSB in Chromatin**—We then tested whether Artemis relied on DNA-PKcs for its damage-induced recruitment as we have found in vitro. Thus, M059J glioblastoma cells that do not express DNA-PKcs (DNA-PKcs-deficient cells, Fus9) and M059J-complemented cells that contain an extra copy of the human gene coding for DNA-PKcs (DNA-PKcs-complemented cells, Fus1) (43) were transfected with a pcDNA1.1 vector expressing the Myc-tagged Artemis protein. After 48-h expression, both cells were treated with Cal, and the recruitment of Artemis to the insoluble chromatin fraction was assessed. As shown in Fig. 6, Artemis was equally expressed from the transfected vector in both DNA-PKcs-proficient and -defective cells, indicating that Artemis is not likely to be stabilized by its interaction with DNA-PKcs (Fig. 6, lanes 1 and 4). After treatment with Cal, Ku80 was similarly recruited to the damaged chromatin in both cell lines, as reported (19), but in sharp contrast, Artemis mobilization to the P2 fraction was only detected in the DNA-PKcs-proficient cells together with DNA-PKcs recruitment, and in addition, it was detected as a phosphorylated form (Fig. 6, compare lanes 3 and 6).

The simplest interpretation of these results is that DNA-PKcs is necessary for Artemis stable recruitment to DSB in chromatin in agreement with our in vitro data.

The Stabilization of Artemis on DSB-containing Chromatin Is Dependent on the Kinase Activity of DNA-PKcs—Since we have set up conditions allowing us to analyze in the cells the DNA-PKcs-dependent mobilization of Artemis to broken chromatin, we then challenged our conclusions from in vitro experiments by assessing in vivo the effect of the DNA-PKcs kinase activity on the association of Artemis with damaged DNA. The selective DNA-PKcs inhibitor NU7026 has been shown to exhibit a strong DNA-PKcs-dependent radiosensitization effect on cells at 10 μM when added 1 h before irradiation (53). Therefore, Guetel-A cells were pretreated or not with 10 μM NU7026 for 1 h, and then Cal was added for further incubation at 37°C. The extraction protocol was achieved as above, and the WCE and P2 protein fractions were analyzed by Western blot. As shown in Fig. 7A, the DNA-PKcs inhibitor had no obvious effect on the strong mobilization of Ku and DNA-PKcs to the damaged chromatin (Fig. 7A, compare lanes 3 and 4). XRCC4 was also heavily recruited under DNA-PK activity permissive or preventive conditions, but NU7026 abolished its phosphorylation (Fig. 7A, compare lanes 3 and 4). Accordingly, we have shown elsewhere that DNA-PKcs-dependent XRCC4 phosphorylation was dispensable for its recruitment to damaged chromatin (19). The lack of XRCC4 phosphorylation in the presence of NU7026 is thus a good indicator of the actual DNA-PKcs inhibition under these conditions. Artemis was clearly mobilized to the P2 frac-

**FIGURE 5.** Analysis of protein fractionation in untreated and calicheamicin-treated Guetel cells. Guetel (Art−) or Guetel-A (Art+) cells in culture dishes were treated or not for 1 h at 37°C 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 S1 and S2 soluble fractions and P2 insoluble material. Protein samples were denatured and separated on SDS-polyacrylamide gels (8% for standard separation or 15% for H2AX, Rad51, and HP1α isolation) followed by electrotransformation on membrane. The membranes were blotted with the antibodies as indicated. Artemis was detected with an anti-Myc antibody. Electrophoresis mobility shift is shown by an arrow. A, cell treatment was with 10 nM Cal, lanes 1 and 2. Guetel-A cells were either not treated (NT), irradiated with UV-C light (UV; 200 J/m²), or treated with cis-diaminedichloroplatinum-II (CDDP; 200 μM) or neocarzinostatin (Ncs; 1 μM) for 1 h at 37°C. B, ATP-dependent DNA-PKcs-proficient cells together with DNA-PKcs recruitment, and in addition, it was detected as a phosphorylated form (Fig. 6, compare lanes 3 and 6).

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**FIGURE 6.** Role of DNA-PKcs in Artemis recruitment to damaged chromatin. DNA-PKcs-deficient (Fus9) and DNA-PKcs-complemented (Fus1) glioblastoma cell lines were transfected with the expression vector for C-terminal Myc-tagged Artemis. 48 h after transfection, Fus9 and Fus1 cells were incubated for 1 h at 37°C with 10 nM Cal in unsupplemented medium. Then cells were collected and lysed in denaturing buffer (WCE) or fractionated by two consecutive extractions as shown in Fig. 5, leading to S1 and S2 soluble fractions and P2 insoluble material. Protein samples were denatured and separated on SDS-polyacrylamide gels (8% for standard separation or 15% for H2AX, Rad51, and HP1α isolation) followed by electrotransformation on membrane. The membranes were blotted with the antibodies as indicated. Artemis was detected with an anti-Myc antibody. Electrophoresis mobility shift is shown by an arrow. A, cell treatment was with 10 nM Cal, lanes 1 and 2. Guetel-A cells were either not treated (NT), irradiated with UV-C light (UV; 200 J/m²), or treated with cis-diaminedichloroplatinum-II (CDDP; 200 μM) or neocarzinostatin (Ncs; 1 μM) for 1 h at 37°C. B, ATP-dependent DNA-PKcs-proficient cells together with DNA-PKcs recruitment, and in addition, it was detected as a phosphorylated form (Fig. 6, compare lanes 3 and 6).

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tion of Cal-treated cells in which it was detected in a phosphorylated form (Fig. 7A, lane 3). In contrast, Artemis was hardly detectable in the P2 fraction of cells treated with both NU7026 and Cal, yielding the same marginal amount as in the control untreated cells (Fig. 7A, compare lanes 2 and 4). When the WCE were analyzed, Artemis was fully phosphorylated in the Cal-treated cells but, after treatment with Cal in the presence of NU7026, migrated as in the control untreated cells (Fig. 7B). This indicates that the phosphorylation observed under these conditions mostly relied on the NU7026-sensitive DNA-PKcs activity. In conclusion, the stabilization of Artemis on DSB is dependent on the kinase activity of DNA-PK.

**DISCUSSION**

Artemis protein is the factor of the NHEJ apparatus for which the least is known about its interactions with the other components of the reaction. Here, we have analyzed the interactions of Artemis and NHEJ proteins both in a context of human nuclear cell extracts and in cells.

In untreated cells, most of Artemis belongs to the soluble nucleoplasmic compartment, since it is exclusively found in the soluble protein fraction, as shown here by biochemical analysis. In contrast, DSB induce the mobilization of Artemis together with DNA-PK and XRCC4/ligase IV proteins to a detergent-resistant nuclear compartment. The Artemis mobilization to damaged chromatin is specifically initiated by DSB-inducing agents, like that of the other NHEJ factors as detected by us (19) and another group using this technique (14, 54). The time course of appearance of this recruitment paralleled that of the kinase activity bound to double-stranded oligonucleotides on beads in cell extracts and also the association of Artemis with DNA-PKcs in co-IP experiments. In addition, experiments in cells corroborated this result, since Artemis recruitment in damaged chromatin was not detected in cells pretreated with a DNA-PKcs-specific inhibitor.

It could be argued that the short DNA targets used in vitro may not permit sufficient space for colocalization of DNA-PKcs, Ku, and Artemis. However, this hypothesis is unlikely, since an excess of the Ku80 C-terminal peptide promoted efficiently Artemis/DNA-PKcs dissociation even in the absence of DNA, implying another mechanism.

Interestingly, Ku dissociates Artemis from DNA-PKcs in the presence of DNA, whereas the C-terminal Ku80 peptide is efficient without DNA. It has been demonstrated that the conserved C-terminal motif of Ku80 is required for the efficient recruitment of DNA-PKcs to DNA ends in vitro (55). In addition, cells expressing a form of Ku80 lacking the C terminus exhibit a DNA-PKcs minus phenotype despite the fact that the kinase is present and the Ku DNA binding property is conserved, indicating that the extreme C terminus of Ku80 is also required for DNA-PKcs recruitment and activation at DNA DSB in cells (48, 55). Moreover, a C-terminal fragment of Ku80 confers a dominant negative effect on DSB repair and radiosensitivity (56), and the Ku80 C-terminal peptide sensitizes cells to DSB (57). Since it has been shown that Ku and DNA-PKcs do not associate in the absence of a DNA terminus (58), whereas a Ku80 C-terminal fragment is sufficient to interact with DNA-PKcs (45), it is most likely that Ku binding to a DNA end exposes the Ku80 C terminus as a docking module for DNA-PKcs, probably necessary for other subsequent stabilizing interactions (55). This region absent in the x-ray structure of the Ku heterodimer with DNA (59) was shown to be flexible in solution by NMR determination and exhibited a high helical propensity, allowing folding upon Ku binding to DNA or to its protein partner (60, 61). Thus, Ku binding to DNA or the Ku80 C-terminal motif may displace Artemis from DNA-PKcs by competition on the same domain of the kinase. Previous studies have...
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established that residues in the FAT domain of DNA-PKcs adjacent to the catalytic domain are involved in its interaction with Ku (62), contrary to the leucine-rich region (54). One possibility is that the DNA-PKcs FAT domain is involved similarly in interactions with Ku80 and Artemis, implying that some domain of Artemis may share a structural homology with the Ku80 C terminus. Another possibility is that Artemis dissociation is elicited by a change of DNA-PKcs conformation. Indeed, several studies have revealed extensive conformational changes of the DNA-bound kinase consisting of domain rearrangements, including the FAT portion to form channels that could accommodate duplex and single-stranded DNA (63–66). Thus, deciphering whether Ku dissociates the Artemis-DNA-PKcs complex via an induced change of DNA-PKcs conformation or a competition on the same binding site on DNA-PKcs awaits further experiments. In addition, the role of another DNA-PKcs partner for this dissociation in cells cannot be excluded.

Our present data from experiments in vitro and in cells showed that the activity of the DNA-PKcs kinase prevented Artemis dissociation from the DNA-PK-DNA complex. This is in agreement with the results of Ma et al., who showed that DNA-PKcs is physically required for Artemis activity after its phosphorylation (42) and that DNA-PKcs remained associated with Artemis immunobeads after phosphorylation and stringent washes (31). Under our conditions, Artemis accumulation on double-stranded oligonucleotides in vitro was correlated with the length of the 5′ or 3′ single-stranded tail. A plausible explanation is that DNA-PKcs activation may be proportional to the length of the single-stranded tail as reported (67) and that the activity level may in turn regulate the extent of the Artemis-DNA-PK complex stabilization. The shift from an unstable Artemis-DNA-PK-DNA complex to a stable association probably relies on a change in the geometry of the protein-DNA complex involving Artemis and/or DNA-PKcs phosphorylation. Indeed, differential autophosphorylation of DNA-PKcs on two major clusters has been shown to greatly influence DNA end access to processing enzymes (reviewed in Ref. 11). A current model is that an intermediate phosphorylated state of DNA-PKcs directs a rearrangement of the DNA-PK complex that ensures access to broken ends, whereas complete autophosphorylation dissociates the complex from DNA ends (14, 15). Thus, the DNA-PKcs associated with Artemis under kinase permissive conditions may be either unphosphorylated or subphosphorylated and may be necessary to load Artemis to its substrate and/or to maintain a proper configuration of the DNA termini. Alternatively, Artemis phosphorylation per se could change its interactions with DNA-PK and/or DNA. Artemis phosphorylation takes place on the C-terminal domain (31, 37), possibly allowing extrusion of the inhibitory C-terminal domain (31), which could also be implicated in maintaining Artemis association with the DNA-PK-DNA complex.

In Cal-treated cells, Artemis was detected as a phosphorylated protein, and a specific DNA-PKcs inhibitor abrogated this phosphorylation, implying that DNA-PKcs was mainly responsible for Artemis phosphorylation under these conditions. This is in contrast with other reports that also implicated ATM in Artemis IR-induced phosphorylation (35, 37). Our results more easily agree with the biochemical evidence of the DNA-PK-mediated activa-

FIGURE 8. Model for DNA-PKcs-Artemis interaction changes in the presence of a DNA double strand break. For clarity, only one end of the DSB is shown. In addition to the interactions shown, a self-association of the DNA end bound DNA-PKcs molecules allows the tethering of the DNA ends during the reaction (1). Under cellular salt conditions, DNA-PKcs-Artemis cannot bind to DNA ends without Ku (2). Ku cannot bind to the DNA-PKcs-Artemis complex without DNA ends (3). Ku binding to a DNA end exposes the Ku80 C terminus as a docking module for DNA-PKcs, and Artemis needs DNA-PKcs for binding to the DNA end (4). The recruitment of DNA-PKcs to the Ku-DNA complex induces an extensive conformational change of DNA-PKcs, and Artemis dissociates under kinase-preventive conditions (e.g. kinase inhibitor, non-activating DNA end) (5). Under kinase-permissive conditions, a supplementary conformational change of partially autophosphorylated DNA-PKcs (and possibly its Ku partner) and/or phosphorylation of Artemis maintains the DNA-PKcs-Artemis association and elicits Artemis endonuclease activity. See “Discussion” for comments and references.

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