Transcriptional activation of DNA-dependent protein kinase catalytic subunit gene expression by oestrogen receptor-α

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INTRODUCTION

In addition to its widespread role in human physiology, oestrogen (E2) is implicated in the development and progression of proliferative disorders such as breast cancer (Deroo & Korach, 2006). E2 exerts its effects through the E2 receptors (ERs) ERα and ERβ. ERα is a member of the class I nuclear hormone receptor superfamily. On ligand binding, the receptor forms homodimers that bind E2-responsive elements (EREs) located in the regulatory region of target genes (Martinez & Wahli, 1989). Although the effects of E2 on the proliferation of human breast cancer cells have been known for many years, only recently have gene expression profiling studies suggested a role of the hormone in DNA repair (Gadal et al., 2005).

DNA-dependent protein kinase (DNA-PK) is a serine/threonine protein kinase comprising a catalytic subunit (DNA-PKcs) and Ku subunits, which act as regulatory elements (Collis et al., 2005). DNA-PK is the main component of the non-homologous end-joining pathway of DNA double-strand break (DSB) repair in mammalian cells (Lees-Miller, 1996). It has been proposed that DNA-PK is a molecular sensor of DNA damage, which enhances DSB repair through the phosphorylation of many downstream targets (Anderson & Lees-Miller, 1992; Kysela et al., 2005; Hah et al., 2007). The crucial role of DNA-PK is to repair DSBs that either arise endogenously during normal cellular processes or are exogenously caused by genotoxic agents such as ionizing radiation (IR). Unrepaired DSBs are known to trigger cell-cycle checkpoint arrest and cell death (Norbury & Hickson, 2001). Recently, we demonstrated that glycogen synthase kinase 3 stabilizes ERα and modulates its transcriptional activity (Medunjanin et al., 2005). In addition, co-immunoprecipitation studies revealed the involvement of a 70 kDa protein that was subsequently identified as Ku70, a component of the DNA-PK holoenzyme. When studying the role of DNA-PK in ERα activity, increased...
DNA-PKcs expression levels were observed in cells treated with E2. We thus tested the hypothesis that DNA-PKcs is a direct target of ERα.

RESULTS

E2-dependent induction of DNA-PKcs

To test our hypothesis, cells from the breast cancer cell line MELN were treated with E2 in a time-dependent manner, which resulted in a marked upregulation of DNA-PKcs, and an expected downregulation of ERα within 6 h (Fig 1A). Quantitative real-time reverse transcription PCR revealed that E2 induced a significant—in about twofold—induction of DNA-PKcs messenger RNA expression that peaked at 1 h (Fig 1B). This was confirmed by transfection of ERα–Flag fusion protein into ERα-negative COS-7 cells yielding upregulation of DNA-PKcs after E2 treatment only in ERα-positive cells (Fig 1C). E2-dependent induction of DNA-PKcs was reduced markedly with either the specific ER antagonist ICI 182,780 (Fig 1D) or the transcriptional repressor actinomycin D (ActD; Fig 1E). Furthermore, we used small interfering RNA (siRNA) to knockdown ERα levels. In the presence of the specific siRNA, DNA-PKcs protein levels were reduced (Fig 1F).

Oestrogen transactivates the DNA-PKcs promoter

Subsequently, the promoter region of DNA-PKcs was analysed for EREs. The 13 bp palindromic ERE with the consensus sequence 5′-GGTCANNNTGACC-3′ is well known (Klein-Hitpass et al., 1988). However, it has been shown that ‘imperfect’ palindromic EREs might also act synergistically to permit translational activity (Martinez & Wahli, 1989). Analysis of the DNA-PKcs promoter region revealed the presence of three EREs. The 13 bp palindromic ERE with the consensus sequence 5′-GGTCANNNTGACC-3′, to which ERα binds, was subsequently restricted to position −220 upstream from the transcriptional activation of DNA-PKcs.

S. Medunjanin et al.
DNA-PKcs initiation site, cloned into the luciferase reporter plasmid and examined for promoter activity 72 h after E2 stimulation, confirming the relevance of the hypothesized EREs in DNA-PKcs transactivation. E2-induced increase in luciferase activity was reduced when the cells had been preincubated with the anti-E2 ICI 182,780 before stimulation (Fig 2C).

Binding of ERα to the DNA-PKcs promoter was further confirmed by chromatin immunoprecipitation assays with ERα antibody. In MELN cells, occupancy of the DNA-PKcs promoter by ERα was not detected in the absence of E2, but increased markedly on E2 stimulation (Fig 2D, upper panel). In subsequent experiments, we amplified a portion of human DNA-PKcs promoter bearing a putative half or imperfect ERE. The ERα binding to these sites was reduced markedly after E2 treatment (Fig 2D). Immunoprecipitations performed with either immunoglobulin G (IgG) or a control region (from −4,014 to −3,717) of the DNA-PKcs promoter upstream from the ERE were used as controls. Together, these findings indicate that: (i) ERα directly binds to the DNA-PKcs promoter and (ii) E2 stimulation is associated with increased occupancy of EREs by ERα.

Oestrogen-induced double-strand break repair

Increased expression of DNA-PKcs alone might provide important protection from genotoxic stress, even in cells already containing functional DNA-PK (Shen et al., 1998). To determine the functional significance of our finding, we examined the effect of E2 on DNA DSB repair in human MELN cells by studying the kinetics of γ-H2AX foci formation, which result from DSBs during irradiation (Sedelnikova et al., 2002; Rothkamm & Lobrich, 2003). Time course experiments revealed that the number of γ-H2AX foci reached its maximum 1 h after irradiation of MELN cells. After irradiation, it fell almost to control level within 6 h (Fig 3A). Foci formation was reduced significantly when cells were exposed to E2 before irradiation (Fig 3A). As foci formation takes a finite
Fig 3: E2-induced DSB repair. (A) MELN cells were grown in phenol red-free medium supplemented with 10% charcoal–dextran-stripped FBS for 3 days. After pretreatment with 100 nM of E2 for 20 h, cells were left untreated or treated with IR (10 Gy). Cells were fixed for immunofluorescent staining with the γ-H2AX antibody (Ser 139) as a marker for DSBs. Foci were counted in at least 50 cells per condition. Error bars represent the s.d. values from at least three independent experiments. Differences were statistically significant at the P < 0.05 level. (B) MELN cells were grown as above. After 2 h pretreatment with 100 nM of E2 for 20 h, cells were exposed to IR (20 Gy) and incubated for different times. The distribution of tail moments among a population of 100 cells for each time point was plotted. Error bars represent the s.d. values from at least three independent experiments. Representative images are shown additionally. Scale bars, 100 μm. (C) MELN cells were treated as in (A) followed by immunoblotting with specific antibodies as indicated. The asterisk represents an unspecific band recognized by the Chk2 antibody. (D) MELN cells were grown in phenol red-free medium supplemented with 10% charcoal–dextran-stripped FBS for 3 days. After pretreatment with 5 μM of NU7026 and 100 nM of E2 for 20 h, cells were treated with IR (10 Gy) and incubated for 2 h. Cell lysates were assayed for expression of proteins with the indicated antibodies. β-Actin was used as a loading control. ATM, ataxia telangiectasia mutated; Chk2, checkpoint kinase 2; DSB, double-strand break; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; E2, oestrogen; ERα, oestrogen receptor-α; FBS, fetal bovine serum; γ-H2AX, phosphorylated histone H2AX; IB, immunoblotting; IR, ionizing radiation; U, untreated.

amount of time, this assay was not able to assess the initial number of DSBs. Thus, DSB induction and repair after IR was additionally visualized and quantified by using the neutral COMET assay. Pretreatment of MELN cells with E2 significantly increased the repair of damaged DNA—that is, decreased the comet tail moment—after irradiation (Fig 3B).

Western blot analyses visualized reduced phosphorylation of γ-H2AX in E2-treated cells compared with untreated cells and confirmed the results of the immunofluorescence microscopy (Fig 3C). A previous study (Li & Stern, 2005) indicated that phosphorylation of DNA repair protein checkpoint kinase 2 (Chk2) at Thr68 depends on the activation of DNA-PK after irradiation. Therefore, phosphorylation of Chk2 at Thr68 was used to show that DNA-PK had indeed been activated. By contrast, phosphorylation of ataxia telangiectasia mutated (ATM), another central kinase of DSB repair, was not sensitive to E2 treatment (Fig 3C). When DNA-PK had been inhibited, E2 lost its preventive effect on γ-H2AX foci formation after IR (Fig 3D). Involvement of ERα in DSB repair was further supported by using the specific ER antagonist ICI 182,780. E2-dependent induction of DNA-PKcs was reduced markedly when MELN cells were preincubated with the anti-E2 ICI 182,780 before irradiation. Consequently, E2-induced increased phosphorylation of Chk2 at Thr68 was prevented markedly in ICI-treated cells (Fig 4A).

Previous studies demonstrated that ATM- and Rad3-related (ATR), another PI3-related kinase, also regulate phosphorylation of Chk2 at Thr68 (Matsuoka et al, 2000; Wang et al, 2006). To rule out ATM and ATR as activators of Chk2, we used the specific ATM/ATR kinase inhibitor CGK733. No reduction in the phosphorylation of Chk2 at Thr68 after E2 treatment was observed when the ATM/ATR inhibitor was used (Fig 4B).

DISCUSSION

In recent years, mammalian DNA-PKcs has been shown to be a crucial component of the signalling mechanisms of both DSB repair and V(D)J recombination (Shen et al, 1998), which was
now, our data identify DNA-PK as the mediator of E2-induced DSB repair. Elevated levels of DNA-PK warrant a quick and strong response to DNA damage, resulting in reduced cellular levels of DSBs. An involvement of ATM, another important DNA repair enzyme, could be excluded in this context.

Clinically, the ERα has a central role in controlling cell growth in mammary tissue, and its expression level in breast cancer is associated with longer survival and a better response to therapeutic measures (Ruiz et al., 2006). However, through DNA-PK activation, ERα positivity might confer a selective advantage to tumour cells when exposed to radiotherapy or chemotherapeutic agents. Consequently, anti-E2s could sensitize these cells for therapies. Indeed, breast cancer cell lines incubated with tamoxifen were shown to be more sensitive to irradiation (Wazer et al., 1989), and the combination of radiotherapy and anti-hormonal therapy has been shown to prevent tumour recurrence (Fisher et al., 2002). Whether anti-hormonal pretreatment before radiotherapy improves treatment results in patients with breast cancer has, however, not yet been established. Nonetheless, our finding of an interaction between two central components of DNA replication and repair sheds further light on tumour biology and reveals new possibilities for the prevention and therapy of E2-sensitive proliferative diseases.

**METHODS**

**Antibodies and reagents.** The following antibodies, kit and inhibitors were used: anti-cathepsin D (4G2) and anti-α-actin (Abcam, Cambridge, UK); anti-ERα (MAB463; Chemicon, Bad Nauheim, Germany); anti-ERβ (HC-20), anti-Ku80 (SC-1484) and anti-DNA-PKcs (sc-5282; Santa Cruz Biotechnology, Heidelberg, Germany); anti-Chk2 (2662), anti-Chk2 (3440) and DNA Damage Sampler Kit (#9947; New England BioLabs, Frankfurt am Main, Germany); alexa-green conjugated anti-mouse IgG (Molecular Probes, Eugene, OR, USA); ICI 182,780, ATOMAT Kinase inhibitor (CGK 733), ActD and DNA-PK inhibitor (NU7026; Merck, Darmstadt, Germany).

**Gene silencing with siRNAs.** Transfection of MELN cells with siRNA has been described before (Grisonaud et al., 2007). The siRNA oligonucleotides with 3′-TT overhangs were purchased from MWG-BIOTECH AG (Ebersberg, Germany). The following siRNA sequences were used: (siERα) 5′-AGGCUCAUUCCAGC CACAGTTGdTdT-3′; and (siDNA-PKcs) 5′-CUUUAUGGGUGGC CAUGAGdTdT-3′. The concentration of siRNAs was 20 nM during transfection. For control, we used GL3-targeted siRNA.

**Comet assays.** The neutral COMET assay (single-cell gel electrophoresis assay) was performed according to the manufacturer’s instructions (Trevigen, Gaithersburg, MD, USA). Briefly, after irradiation, cells were collected and mixed with low-melting agarose. After lysis, electrophoresis was performed at 1 V/cm and 15 mA for 40 min. Slides were stained with SYBR Green dye (Invitrogen, Karlsruhe, Germany) for 10 min. A total of 100 randomly selected cells per sample were captured under a Zeiss fluorescent microscope and digital fluorescent images were obtained using the Axiolab software (Carl Zeiss Imaging, Jena, Germany). The relative length and intensity of SYBR green-stained DNA tails to heads were proportional to the amount of DNA damage present in the individual nucleus and was measured by Olive tail moment with TriTek Comet Score software (TriTek, Sumerduck, VA, USA).
Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays were performed as described previously (Wei et al., 2006). Briefly, lysates were sonicated with Branson Sonifer Cell Disruptor B15 (Branson, Danbury, CT, USA) to shear DNA to an average size of 600–1,000 bp. For chromatin immunoprecipitation, anti-ErRz (MAB463) and IgG (Pierce, Bonn, Germany) were used. Purified DNA was amplified across the DNA-PKcs promoter region.

Luciferase assay. The MELN cells were washed with phosphate-buffered saline (Mg$^{2+}$ and Ca$^{2+}$ free) and lysed in 150 μl per well luciferase cell culture lysis reagent (Promega, Mannheim, Germany). Luciferase assays were performed by using the firefly luciferase assay system from Promega according to the manufacturer’s instructions and quantified with a luminometer (LB9506, Berthold, Bad Wildbad, Germany).

Plasmids. The plasmids for ErRz have been described previously (Medunjanin et al., 2005). Constructs of the DNA-PKcs promoter (GenBank accession number U63630) were produced by PCR amplification using genomic DNA from MELN cells. The resulting fragments, spanning the promoter region from positions −1,026, −606, −220 to position −2 relative to the transcriptional start site, were cloned into the XhoI and Xhol cloning sites of the pG3-LucBasic vector upstream from the firefly luciferase gene (Promega).

Statistical analysis. Data are presented as mean ± s.e.m. Statistical analysis was performed by analysis of variance. Post-test multiple comparisons were performed by using the Bonferroni method. All experiments were independently repeated at least three times. Additional experimental procedures are described in the supplementary information online.

Supplementary information is available at EMBO reports online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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