Staurosporine-sensitive protein phosphorylation is required for postreplication DNA repair in human cells

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Abstract DNA repair is an important factor of stability of pro- and eukaryotic genomes which plays a central role in mutagenesis and carcinogenesis. Genetic control of nucleotide excision repair (NER) in mammalian cells is well studied, but little is known about molecular mechanisms of postreplication repair (PRR) which allows bypass of base lesions in template strands after DNA replication. In *Saccharomyces cerevisiae* PRR is controlled by the *RAD6/RAD18* pathway which involves *POL30* gene encoding proliferating cell nuclear antigen (PCNA), and in human cells PCNA is known to be closely associated with the newly replicated chromatin where PRR probably takes place. In UV-irradiated human cells distinct PCNA foci may be detected in some cells which accumulate phosphorylated breast cancer susceptibility protein BRCA1 and another protein BARD1. Human PCNA is also known to be phosphorylated after UV-irradiation. In this study we found that the known inhibitor of protein kinases staurosporine supresses PRR in NER-deficient cells which is consistent with the view that BRCA1 and PCNA are required for PRR. We also have shown that the distinct PCNA foci in UV-irradiated NER-deficient cells are actually associated with the newly replicated chromatin. Since RAD18 protein is not essential for normal DNA replication and directly controls PRR in yeast, we analysed whether this protein as well as its human homologs (HR18A and HR18B) have common domains with BRCA1 and BARD1. It is found that HR18A has a subregion of homology to BARD1 and HR18A-to BRCA1. Taken together the results indicate that BRCA1 and BARD1 may be involved in PRR in human cells.

Key words: Protein phosphorylation; Ultraviolet irradiation; Postreplication repair; Newly replicated chromatin; Proliferating cell nuclear antigen

1. Introduction

Ultraviolet (UV) irradiation of living cells introduces stable chemical lesions into DNA which interfere with its functions and, for cell survival, should be either eliminated or tolerated (bypassed). One well studied mechanism of elimination of the lesions from mammalian genomes is nucleotide excision repair (NER) involving nucleolytic excision of damaged DNA segments followed by local resynthesis performed by specific multiprotein complexes before DNA replication [1,2]. Genetic control of NER is well studied and several human diseases are identified caused by mutations in NER genes, e.g. xeroderma pigmentosum (XP) or Cockayne syndrome [1]. However, some unexcised lesions may enter S-phase, and normal replication machinery cannot copy damaged DNA leaving single-strand gaps in daughter strands opposite the lesions which are then slowly repaired without elimination of the lesions from template strands [3,4]. This process of filling of the daughter-strand gaps (DSG) opposite the lesions in the newly replicated chromatin and called postreplication repair (PRR) is thought to be the main source of the DNA damage-induced mutations but, surprisingly, no gene was identified so far controlling PRR in mammalian cells. In *Saccharomyces cerevisiae* PRR is controlled by the *RAD6/RAD18* pathway which involves proliferating cell nuclear antigen (PCNA) and DNA polymerase δ [5–7]. Two mammalian homologs of the yeast *RAD6* gene (*HR6A and HR6B*) were identified and inactivation of one them (*HR6B*) in mice was shown to lead to male sterility but no defect of PRR was found [8]. Double *HR6A/HR6B* knockout mice or stem cells were not isolated indicating that activity of at least one of the genes is essential for cell viability. Some xeroderma pigmentosum (XP) cells (XP variant) show defects in PRR [9,10] but an underlying genetic defect is not yet known.

PRR is taking place in the newly replicated chromatin during 2–3 h following formation of DSG in S-phase human cells [9,10]. Replication foci in normally proliferating mammalian cells are known to contain tightly bound DNA polymerase δ accessory factor PCNA [11–13]. As was shown earlier [14] replication foci co-localize with chromatin assembly factor 1 (CAF-1) and, therefore, mark the newly replicated chromatin. In UV-irradiated human cells PCNA is known to be phosphorylated and the phosphorylation is inhibited by staurosporine [15]. It has also been shown recently [16] that in UV-irradiated NER-proficient human cells the major breast cancer susceptibility protein BRCA1 also becomes phosphorylated and relocates to the nuclear foci containing PCNA. Human homologs of the yeast RAD51 and protein BARD1 show similar behavior and the results indicate that BRCA1/RAD51/BARD1 may be directly involved in DNA repair functioning as 'caretaker' of the genome integrity [17]. It was suggested [16] that after UV-irradiation phosphorylated BRCA1 mainly relocates to the newly replicated chromatin of S-phase cells, but in the NER-proficient cells used in this study [16] PCNA foci may also be associated with non-S-phase NER sites [18], or with non-NER non-S-phase sites accumulating p21 (Cip1, WAF-1) protein [19].

In this study we have shown that the repair of DSG in completely NER-deficient cells may be inhibited by staurosporine and that distinct PCNA foci in UV-irradiated NER-deficient cells are actually associated with the newly replicated chromatin. BARD1 and BRCA1 proteins are also shown to

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contain regions of statistically significant homology to human homologs of the yeast RAD18 protein HR18A and HR18B, respectively. Taken together, the results suggest that BRCA1 and BARD1 may be directly involved in PRR in human cells.

2. Materials and methods

2.1. Nuclear foci in NER-deficient human cells

The cells (xeroderma pigmentosum complementation group A XP25RO primary fibroblasts) were grown in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal bovine serum on microscopic glass slides in Petri dishes. Two hours before UV-irradiation 5-fluorodeoxyuridine was added to 0.01 mM final concentration. After washing with phosphate-buffered saline (PBS) cells were UV-irradiated (254 nm, 30 J/m^2) and incubated for 1 h in the growth medium containing 0.01 mM 5-iododeoxyuridine (IdU). After washing with PBS, the slides were treated for 2 min at room temperature (RT) with 0.1% Triton X-100 in PBS, washed again with PBS, fixed for 10 min in ice-cold 4% formaldehyde solution in PBS, kept in 80% ethanol at 4°C overnight, dehydrated in ethanol and dried. After incubation in PBS for 15 min at RT, slides were again treated with Triton X-100 (0.1% solution in PBS, 2 min at RT), washed two times with PBS, incubated in 1% blocking reagent (Boehringer) in PBS with 0.02% Tween 20 for 15 min at 37°C and rinsed with 0.5% blocking reagent with 0.02% Tween 20. All dilutions of antibodies and washes were in the same solution and incubations were at 37°C. Then slides were sequentially treated with mouse monoclonal anti-PCNA antibodies (1:30, Oncor), 1 h; biotinylated sheep-anti-mouse IgG (1:100, Sigma), 40 min; avidin-Texas Red (1:50, Vector), 40 min; goat-anti-mouse non-tagged IgG (1:20, Sigma), 1.5 h (to block the mouse epitopes); 4 N HCl, 30 min at RT; monoclonal mouse antibodies against 5-bromodeoxydine which react with IdU (clone isolated by Dr. M. Filatov, 1:50), 60 min; rabbit-anti-mouse IgG (1:50, Sigma), 40 min; and FITC-tagged goat-anti-rabbit IgG (1:100, Miles), 40 min. Then slides were mounted in antifade solution (50 mg/ml propylgallate in glycerol). Conventional fluorescent microscopy was done using an Opton ICM-405 inverted epifluorescence microscope equipped with appropriate objective and filters.

2.2. Analysis of postreplication DNA repair

The cells (SV40-transformed XP12RO cell line) were grown as in Fig. 1. XP12RO cells contain the same mutations in the *XPA* gene as XP25RO cells. After washing in PBS and UV-irradiation (5 J/m^2, 254 nm) cells were incubated for 30 min at 37°C in the growth medium with 5% serum containing [^{3}H]thymidine (20 μCi/ml, 16 Ci/mmol), and then for 3 h in non-radioactive DMEM with 10% serum. Staurosporine (200 nM) was added to growth medium 30 min before UV-irradiation and was also present in the medium during subsequent incubations. The cells were then washed in cold isotonic EDTA, collected using a rubber policeman and placed (10^5 cells in 0.1 ml) on the top of an alkaline sucrose gradient (5–30% in 0.9 M NaCl, 0.3 M NaOH, 10 mM EDTA) overlayed with 0.1 ml of the lysis buffer (0.6 M NaOH, 0.2 M NaCl, 1% sarcosyl, 20 mM EDTA, 0.5 μg/ml proteinase K). The gradients were centrifuged at 30 000 rpm for 90 min at 20°C in an SW-50.1 rotor of an L2-65K Beckman ultracentrifuge. Fractions were then collected on filter paper discs which were washed in cold 5% trichloroacetic acid, ethanol and radioactivity was measured in a liquid scintillation counter.

3. Results and discussion

Fig. 1 shows sedimentation profiles in alkaline sucrose gradients of the newly synthesized DNA in NER-deficient cells treated or not treated with staurosporine. After 30 min labeling with [^{3}H]thymidine of the non-irradiated or UV-irradiated cells they were incubated for 3 h in growth medium, then lysed and sedimented. Control non-irradiated cells show only fastly sedimenting DNA independently of the presence of staurosporine but after UV-irradiation staurosporine was found to inhibit repair of daughter-strand gaps. Total number of the acid-insoluble counts was about the same in all variants

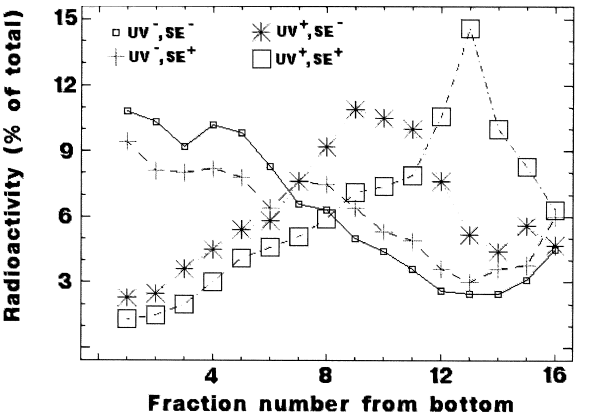

Fig. 1. Inhibition of postreplication DNA repair in XP12RO cells (SV40-transformed cell line) by staurosporine. For experimental details see Section 2. Total tritium counts recovered from gradients were: 71 000 (plus), 56 000 (small squares), 99 600 (stars) and 84 400 (big squares). SE, staurosporine; UV, UV-irradiation, 254 nm, 5 J/m^2.

(see legend to Fig. 1) indicating that UV-irradiation did not repress DNA synthesis and the drug did not induce DNA degradation (apoptosis) under our experimental conditions. Since staurosporine inhibits most protein kinases by mimicking several aspects of adenosine binding [20], our results suggest that phosphorylation of a repair protein is required for PDR in NER-deficient human cells.

At least three relevant proteins are known to be phosphorylated in UV-damaged cells – PCNA [15], p34 subunit of replication factor A (p34-RPA) [21] and BRCA1 [16]. Hyperphosphorylation of p34-RPA correlates with UV-induced loss of activity of the DNA replication complex and was suggested to play a role in DNA synthesis arrest [21] but a possible role of this protein in PRR is not established. PCNA is known to be involved in PRR in *Saccharomyces cerevisiae*

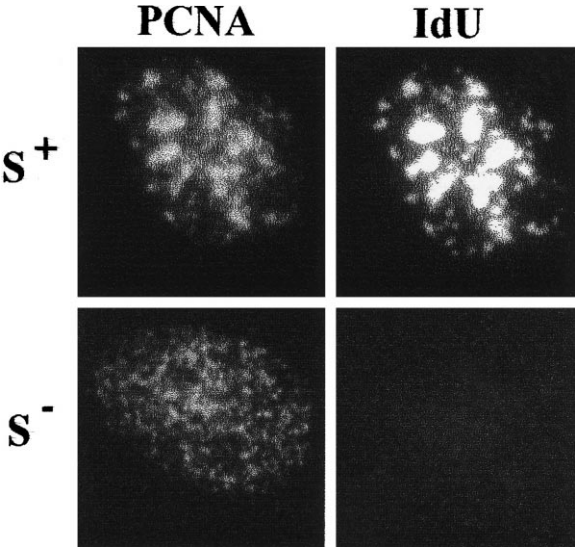

Fig. 2. Two types of nuclear foci containing tightly-bound PCNA in UV-irradiated NER-deficient cells. Two UV-irradiated (30 J/m^2, 254 nm) XP25RO cells (primary fibroblasts) doubly labeled for Triton-unextractable PCNA (left) and incorporated 5′-iododeoxyuridine (IdU) are shown. S-phase cells (S^+, top) incorporate large amounts of IdU into big replication foci, and non-S-phase cells (S^-, bottom) do not incorporate IdU at all.

Fig. 3. Alignment of N-terminal sequences of some mammalian proteins having homology to the yeast RAD18 protein. Sequences of the yeast RAD18 and human BRCA1 and BARD1 proteins are from GenBank; sequences of HR18A and HR18B were produced by conceptual translation of expressed sequences of the public dbEST database (accession numbers AA311754 and AA313966, respectively).

[5,6] and in non-irradiated mammalian cells p34-RPA and PCNA co-localizes in nuclei with newly replicated chromatin [11–14,22] but the relation of the proteins to newly replicated chromatin in UV-irradiated cells is unknown. In non-irradiated S-phase and G2 cells BRCA1 is located in distinct nuclear foci which do not coincide during S-phase with newly replicated chromatin and PCNA [16]. After UV-irradiation of NER-proficient cells BRCA1 translocates to nuclear foci also containing PCNA and it was suggested that the foci represent newly replicated chromatin [16]. However, in UV-irradiated NER-proficient cells PCNA foci may also be associated with non-S-phase NER sites [18] as well as with non-NER non-S-phase sites containing p21 (WAF1/Cip1) [19]. Therefore, it remained unclear whether in UV-irradiated cells phosphorylated BRCA1 actually associates with newly replicated chromatin.

We studied this question by simultaneous double labeling of the UV-irradiated NER-deficient cells for newly replicated chromatin (incorporated 5-iododeoxyuridine, IdU) and PCNA. The results presented in Fig. 2 indicate that after UV-irradiation of XP25RO cultures two subpopulations of cells containing tightly bound PCNA may be seen: one represents S-phase cells incorporating IdU with big distinct foci (Fig. 2, top), and the other non-S-phase cells showing more dispersed and uniform PCNA staining (Fig. 2, bottom). This result suggests that after UV-irradiation tightly bound PCNA does not disappear from big foci of newly replicated chromatin of S-phase cells. Since similar big and distinct PCNA foci accumulating BRCA1 protein were observed in UV-irradiated cells [16] we believe that phosphorylated BRCA1 actually translocates to newly replicated chromatin as was suggested earlier [16]. Dispersed PCNA staining in non-S-phase cells may be indicative of base excision repair of minor UV lesions [19] which requires PCNA [23].

It is likely that in UV-irradiated S-phase human cells tight association of PCNA with newly replicated chromatin reflects direct involvement of this protein in PDR. This protein may be involved in the translesion DNA synthesis in daughter-strand gaps [4,24] stabilized by a mammalian analog of the yeast RAD6/RAD18 complex [7], and co-localization of PCNA with BRCA1 in UV-irradiated S-phase cells [16] may be indicative of possible involvement of BRCA1 in PRR. The UV-induced PCNA/BRCA1 positive foci also accumulate [16] BRCA1-associated RING domain protein, BARD1 [25]. Both BRCA1 and BARD1 contain conserved C-terminal domain (BRCT) which is thought to be involved in DNA damage-responsive checkpoints [26] but the domain is also present in some DNA repair proteins including yeast REV1 protein [26,27] which requires deoxycytidyl transferase for mutagenic bypass of abasic sites in template DNA [28]. *REV1* gene is a member of the *RAD6* epistasis group controlling PRR after UV-irradiation of yeast cells [5–7]. Yeast RAD18 protein [7] which is not involved in DNA damage checkpoints in yeast [29] as well as mammalian BRCA1 and BARD1 proteins [25] are known to contain N-terminal RING domain (C3HC4) which may be responsible for DNA-binding activity of the proteins. In this study we examined whether the two human homologs of yeast RAD18 protein present in dbEST database (HR18A and HR18B, accession numbers AA311754 and AA313966, respectively) are homologous to BRCA1 and BARD1. We found that HR18A is statistically significant homologous to the RING finger region of BARD1 (Fig. 3A) and HR18B is homologous to a similar region of BRCA1 (Fig. 3B) indicating that the two proteins may have common origin and function. Similarity of DNA-binding RING domains of BRCA1, RAD18 and HR18B proteins is consistent with the view that the targeting of phosphorylated BRCA1 to the newly replicated chromatin in UV-irradiated cells [16] may be driven by the affinity of its RING domain to the long-living daughter-strand gaps opposite UV-lesions. In this connection it may be noted that deficiency in DNA repair is detected in some human familial breast cancer cells [30] but it remains to be established whether the cells have mutations in the *BRCA1* gene.

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References

[1] Weeda, G., Hoeijmakers, J.H.J. and Bootsma, D. (1994) BioEssays 15, 249–258.
[2] Lehmann, A.R. (1995) Trends Biochem. 20, 402–405.
[3] Lehmann, A.R. (1979) Nucleic Acids Res. 7, 1901–1912.
[4] Spivak, G. and Hanawalt, P.C. (1992) Biochemistry 31, 6794–6800.
[5] Torres-Ramos, C.A., Yoder, B.L., Burgers, P.M., Prakash, S. and Prakash, L. (1996) Proc. Natl. Acad. Sci. USA 93, 9676–9681.
[6] Torres-Ramos, C.A., Prakash, S. and Prakash, L. (1997) J. Biol. Chem. 272, 25445–25448.
[7] Baily, V., Lauder, S., Prakash, S. and Prakash, L. (1997) J. Biol. Chem. 272, 23360–23365.
[8] Roest, H.P., van Klaveren, J., de Wit, J., van Gurp, C.G., Koken, M.H., Vermey, M., van Roijen, J.H., Hoogerbrugge, J.W., Vreeburg, J.T., Baarends, W.M., Bootsma, D., Grootegoed, J.A. and Hoeijmakers, J.H. (1996) Cell 86, 799–810.
[9] Boyer, J.C., Kaufmann, W.K., Brylawski, B.P. and Cordeiro-Stone, M. (1990) Cancer Res. 50, 2593–2598.

[10] D'Ambrosio, S.M. and Setlow, R.B. (1978) Cancer Res. 38, 1147–1153.
[11] Madsen, P. and Celis, J.E. (1985) FEBS Lett. 193, 5–11.
[12] Bravo, R. and MacDonald-Bravo, H. (1987) J. Cell Biol. 105, 1549–1554.
[13] Toschi, L. and Bravo, R. (1988) J. Cell Biol. 107, 1623–1628.
[14] Krude, T. (1995) Exp. Cell Res. 220, 304–311.
[15] Prosperi, E., Stivala, L.A., Sala, E., Scovassi, A.I. and Bianchi, L. (1993) Exp. Cell Res. 205, 320–325.
[16] Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoekstra, M., Feunteun, J. and Livingston, D.M. (1997) Cell 90, 425–435.
[17] Kinzler, K.W. and Vogelstein, B. (1997) Nature 386, 761–763.
[18] Aboussekhra, A. and Wood, R.D. (1995) Exp. Cell Res. 221, 326–332.
[19] Li, R., Hannon, G.J., Beach, D. and Stillman, B. (1996) Curr. Biol. 6, 189–199.
[20] Prade, L., Engh, R.A., Girod, A., Kinzel, V., Huber, R. and Bossemeyer, D. (1997) Structure 5, 1627–1637.
[21] Carty, M.P., Zernik-Kobak, M., McGrath, S. and Dixon, K. (1994) EMBO J. 13, 2114–2123.
[22] Cardoso, M.C., Leonhardt, H. and Nadal-Ginard, B. (1993) Cell 74, 979–992.
[23] Biade, S., Sobol, R.W., Wilson, S.H. and Matsumoto, Y. (1998) J. Biol. Chem. 273, 898–902.
[24] O'Day, C.L., Burgers, P.M. and Taylor, J.S. (1992) Nucleic Acids Res. 20, 5403–5406.
[25] Wu, L.C., Wang, Z.W., Tsan, J.T., Spillman, M.A., Phung, A., Xu, X.L., Yang, M.C., Hwang, L.Y., Bowcock, A.M. and Baer, R. (1996) Nat. Genet. 14, 430–440.
[26] Bork, P., Hofmann, K., Bucher, P., Neuwald, A.F., Altschul, S.F. and Koonin, E.V. (1997) FASEB J. 11, 68–76.
[27] Callebaut, I. and Mornon, J.P. (1997) FEBS Lett. 400, 25–30.
[28] Nelson, J.R., Lawrence, C.W. and Hinkle, D.C. (1997) Nature 382, 729–731.
[29] Paulovich, A.G., Margulies, R.U., Garvik, B.M. and Hartwell, L.H. (1997) Genetics 145, 45–62.
[30] Parshad, R., Price, F.M., Bohr, V.A., Cowans, K.H., Zujewski, J.A. and Sanford, K.K. (1996) Br. J. Cancer 74, 1–5.