Interference of papillomavirus E6 protein with single-strand break repair by interaction with XRCC1

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XRCC1 protein is required for the repair of DNA single-strand breaks and genetic stability, and is essential for viability in mammals. XRCC1 functions as a scaffold protein by interacting and modulating polypeptide components of the single-strand break repair machinery, including AP endonuclease-1, DNA ligase III α , poly (ADP-ribose) polymerase, DNA polymerase β and human polynucleotide kinase. We show here that the E6 protein of human papillomavirus type 1, 8 and 16 directly binds XRCC1. When tested in CHO derived XRCC1 'knock out' EM9 cells, co-expression of human papillomavirus 16 E6 with human XRCC1 reduced the ability of the latter protein to correct the methyl methane sulfate sensitivity of XRCC1 mutant CHO cell line EM9. These data identify a novel link between small DNA tumour viruses and DNA repair pathways, and suggest a novel explanation for the development of genomic instability in tissue cells persistently infected with papillomaviruses.

Keywords: ber/DNA repair/HPV E6/strand break repair/XRCC1

Introduction

Inherited or acquired deficiencies in DNA repair pathways contribute significantly to the onset of cancer and other human diseases. Single-strand breaks (SSBs) are the most abundant lesions occurring in cellular DNA, with a frequency of 1.5×10^5 per cell per day in human cells (Beckmann and Ames, 1997; Ward 1998), and can be converted into double-strand breaks during DNA replication, potentially resulting in chromosome instability and cell death. One polypeptide implicated in the repair of a broad range of SSBs, including those arising directly from damage to DNA sugars and those arising indirectly from the excision repair of damaged bases, is XRCC1 (Caldecott, 2001). Consequently, cells lacking XRCC1 are hypersensitive to ionizing radiation, hydrogen peroxide, camptothecin and alkylating agents (Thompson and West, 2000). The importance of XRCC1 to genetic stability is indicated by an elevated frequency of spontaneous chromosome aberrations and deletions in *XRCC1* mutant cells, such as the CHO cell-line EM9, and by the

embryonic lethality of *XRCC1*^{-/-} mice (Tebbs *et al.*, 1999; Thompson and West, 2000). In addition, a genetic polymorphism has been identified in human *XRCC1* that may be associated with elevated somatic mutation and cancer risk (Shen *et al.*, 1998; Lunn *et al.*, 1999; Sturgis *et al.*, 1999; Divine *et al.*, 2001; Nelson *et al.*, 2002).

Here, we have employed human papillomavirus (HPV) type 8 E6 as bait in a yeast two-hybrid screen to identify novel cellular proteins with which E6 interacts. HPVs consist of a large group of small DNA viruses, which replicate and cause disease in epithelial surfaces, both mucosal and cutaneous (Stubenrauch and Laimins, 1999). Papillomaviruses causing mucosal disease can be grouped into high- and low-risk types, with HPV6 as the most common low-risk type usually found in benign condylomas, and HPV16 as the most common high-risk type associated with 50% of all cervical cancers (Bosch *et al.*, 1995; zur Hausen, 1999, 2000). For the larger group of cutaneous papillomaviruses affecting keratinizing skin, no type-specific epidemiological risk association has been defined yet. The E6 protein is ~150 amino acids in length, has a molecular weight of 18 kDa and encompasses two C-X-X-C motifs, which form two zinc fingers that are important for many of the properties of the protein described. It has been recognized as a potent oncogene that is associated with a number of events resulting in the malignant conversion of high-risk HPV-infected cells. To overcome antiviral mechanisms of the cell, the high-risk E6 protein targets a variety of cellular proteins involved in regulating cellular defense (Mantovani and Banks, 2001). One of the best characterized activities of E6 from the high-risk viruses is the binding to p53 through a trimeric complex made up of E6, p53 and the E6-associated protein (E6-AP), which is a ubiquitin ligase (Scheffner *et al.*, 1990). This causes the rapid degradation of p53 via the proteasome pathway, a task that is usually performed by mdm-2 in cells not infected by high-risk papillomaviruses (Honda *et al.*, 1997). The E6 protein of low-risk HPV6 does not bind efficiently to p53 and does not cause its degradation, whereas the E6 proteins of cutaneous HPV types 1 and 8 neither bind nor affect the stability or transcriptional activity of p53 (Elbel *et al.*, 1997; Mantovani and Banks, 2001). We demonstrate here that the E6 proteins of HPV1, HPV8 and HPV16, but not of HPV6, bind to XRCC1 and thereby reduce the efficiency of single-strand break repair (SSBR).

Results

HPV1, HPV8 and HPV16 E6 interact with XRCC1 protein by yeast two-hybrid analysis

Yeast Y190 cells harbouring the two-hybrid construct pAS2-HPV8 E6 (Elbel *et al.*, 1997) were employed to screen a cDNA library that was established from HT3

Fig. 1. Interaction between HPV E6 proteins and XRCC1 in the yeast two-hybrid system. **(A)** Y190 cells were co-transformed with GAL4 DBD E6 fusion protein expression vectors and with an expression vector encoding full-length human XRCC1 fused to the GAL4AD (dark bars). Negative controls are empty expression vectors alone (DBD) or in combination (DBD + AD). Positive controls are the known interaction between SV40 large T antigen (SV40 LT) and murine p53 (p53) as well as the full-length GAL4 transcription factor (GAL4). **(B)** Western blotting of co-transformed yeast cells expressing HPV E6 proteins. Yeast cell extracts were separated by 15% SDS-PAGE and blotted onto nitrocellulose filter membrane. Fusion proteins of the GAL4 DBD and the different E6 proteins, which contain in addition a HA epitope of nine amino acids in length, were detected with a monoclonal antibody against HA.

cervical cancer cells, for genes encoding E6-interacting proteins. From 1.6×10^6 transformants, 302 clones were obtained that displayed histidine prototrophy and β -galactosidase activity. Of these, 48 clones were retested by the quantitative ONPG (*o*-nitrophenyl- β -D-galactopyranoside) assay (Elbel *et al.*, 1997), and the resulting nine clones revealing the highest β -galactosidase activity were regrown on leucine-free, but tryptophan-enriched, selection medium containing cycloheximide (10 μ g/ml) to select for yeast clones that had lost the pAS2 bait plasmid. Of the resulting nine clones, sequencing revealed three genes encoding unknown proteins, three encoding rRNA, one encoding the cellular proto-oncogene *junD*, one a heat shock protein hsp-70-related protein and the final one, denoted clone 9, amino acids 107–337 of the XRCC1 protein. Loss of pAS2-HPV8 E6 from clone 9, by selection for growth in the presence of cycloheximide, resulted in a corresponding loss of the His⁺/ β -gal⁺ phenotype, which could be rescued by retransformation with pAS2-HPV8 E6. The same phenotype could also be obtained by co-transformation of Y190 cells with a full-length clone for XRCC1 (pGAD424-XRCC1), HPV1 E6 and HPV16 E6, but not with HPV6 E6 (Figure 1A). Immunoblotting confirmed that the yeast clones employed expressed the expected E6 proteins (Figure 1B).

Fig. 2. Co-immunoprecipitation of HPV E6 proteins and XRCC1 **(A)** Western blot of protein extracts prepared from EM9 cells and EM9 cells stably transfected with a human XRCC1 expression vector (EM9-XH). For detection of the XRCC1 protein, we used the monoclonal antibody mAb33-2-5 directed against XRCC1. *In vitro* translated XRCC1 was employed as a positive control. **(B)** Radioactively labelled *in vitro* translated E6 proteins were mixed with extracts from EM9-XH cells (II–IV) and from EM9 cells (V) as a control, and immunoprecipitated with no antibody (IV) or with a monoclonal antibody directed against XRCC1 (II and V) or GAL4 (III). The top of the figure (I) shows 10% of the input of the labelled E6 translation mixtures.

Binding of E6 to XRCC1 in mammalian cell extracts

To verify the results obtained in the yeast two-hybrid system, we performed co-immunoprecipitation experiments with *in vitro* translated and radioactively labelled E6 proteins. As a source for human XRCC1, we used cellular extracts from EM9-XH CHO cells, and those from the parental EM9 cells as control. EM9 cells are a subclone of wild-type AA8 CHO cells (Thompson *et al.*, 1980, 1982) that carry a frameshift mutation in XRCC1 resulting in an XRCC1 null mutation (Shen *et al.*, 1998; Tebbs *et al.*, 1999). In the EM9-XH cell line, the XRCC1 null mutation has been fully complemented by stable overexpression of human XRCC1 (Thompson *et al.*, 1990; Caldecott *et al.*, 1992, 1995). Western blot analysis of extracts from both EM9 and EM9-XH cells with a monoclonal antibody directed against XRCC1 confirmed the presence of XRCC1 in EM9-XH cells (Figure 2A). To test for co-immunoprecipitation, the same amount of cellular protein as that used for immunoblotting was incubated with equivalent aliquots of *in vitro* labelled E6. As shown in Figure 2B, II, the E6 proteins of HPV1, 8 and 16 were immunoprecipitated with XRCC1-specific antibody, whereas HPV6 E6, even from two different HPV6 subtypes (HPV6a and HPV6b; Grassmann *et al.*, 1996),

Fig. 3. Far western blot for protein-protein interaction. (A) To identify a direct interaction with the XRCC1 protein, the prokaryotic expressed E6 protein of HPV16, DNA ligase III α as a positive control, as well as HPV16 E2 as a negative control, were separated by SDS-PAGE, transferred to nitrocellulose and stepwise renatured. 32 P-labelled prokaryotic expressed XRCC1 protein was incubated with the membrane, as described in Materials and methods, and XRCC1-binding proteins identified by autoradiography. (B) Western blot analysis of E6 and XRCC1 mutant proteins. Equal amounts of XRCC1_402-633 and XRCC1_1-170 protein extracts (as estimated by Coomassie Blue-stained gels) and an aliquot of affinity-purified T7-tagged HPV16 E6 protein (E6) were separated by 15% SDS-PAGE and transferred to nitrocellulose. The membrane was treated identically to the blot presented in (C) and received only anti T7-HRP antibody, but was not incubated with E6 protein. (C) Far western analysis of the interaction of XRCC1 mutant proteins with E6. Separation and transfer of proteins were performed as in (B) and as described in Materials and methods. The blot was incubated overnight with recombinant T7-tagged HPV16 E6 protein. Bound E6 was detected by a T7-HRP antibody and chemiluminescence. (D) Western blot analysis with a polyclonal anti-XRCC1 antiserum. The membrane shown in (D) was reprobed with a polyclonal antiserum directed against human XRCC1. Molecular weight markers in kDa are shown on the right of each membrane.

did not interact with XRCC1. These data fully confirm the results obtained by yeast two-hybrid analysis.

Interestingly, a clear difference in the ability of the different E6 proteins to bind XRCC1 was observed, with HPV16 E6 as the strongest binding protein, followed by HPV1 E6 (20% binding affinity relative to HPV16 E6) and then HPV8 E6 (11% relative binding affinity). To verify the interaction between HPV16 E6 and XRCC1, we performed a far western blot with prokaryotic expressed 32 P-labelled XRCC1 protein to probe prokaryotic expressed HPV16 E6, along with DNA ligase III α as positive control and HPV16 E2 as negative control (Figure 3A). In addition to the known interaction of XRCC1 with DNA ligase III α (Caldecott *et al.*, 1996), we observed binding of XRCC1 to HPV16 E6 by far western blotting, confirming that XRCC1 protein and E6 protein interact directly with each other. To further map the interaction domain of XRCC1 involved in binding to E6, additional far western experiments were performed. Truncated XRCC1 proteins XRCC1_1-170 and XRCC1_402-633, encompassing amino acid residues 1-170 or 402-633, respectively, were expressed in *Escherichia coli* as His-tagged fusion proteins and affinity purified. Equal amounts of both proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with highly purified, soluble T7-tagged HPV16 E6 protein, which was demonstrated to be biologically active in an *in vitro* p53-degradation assay (data not shown). E6 only bound to XRCC1_1-170 and not to XRCC1_402-633 (Figure 3C). Control experiments revealed that this

Fig. 4. Clonal survival of EM9 cells transfected with XRCC1 and HPV16 E6 after MMS treatment. EM9 cells were treated for 1 h with 0.3 mM MMS after transfection with 100 ng of expression vector for human XRCC1 (pCD2EXH) and 1 μ g (1), 3 μ g (2) or 5 μ g (3) of empty expression vector pLXSN (hatched bars) or expression vector for HPV16 E6 (stippled bars). After 48 h, cells were fixed and surviving clones were counted in four different areas of each tissue culture dish. Each bar represents the average of clones counted from eight independent experiments. Standard deviations are given as vertical lines within each bar.

binding was specific and not due to cross-reactivity of the XRCC1_1-170 protein with the anti-T7 antibody used for detection of E6, as no signal was obtained in the absence of added E6 protein (Figure 3B). Furthermore, the T7 antibody reacted with a single band corresponding to E6 in the E6 preparation used for the far western blotting experiment, which excludes the possibility that the protein bound to XRCC1_1-170 is a protein unrelated to E6. Reprobing of the membrane with a polyclonal XRCC1 antibody resulted in bands corresponding to proteins with an apparent molecular weight of 42 kDa for XRCC1_402-633 and 34 kDa for XRCC1_1-170 (Figure 3D). The different signal intensities of XRCC1_402-633 and XRCC1_1-170 are most likely due to the use of a polyclonal antiserum. In both protein preparations, an additional band of lower molecular weight than the truncated XRCC1 proteins can be seen, which is either a degradation product of the different XRCC1 proteins or a contaminant *E.coli* protein cross-reacting with the antibody (Figure 3D). Taken together, these results demonstrate that HPV16 E6 specifically interacts with the N-terminus of XRCC1. In combination with the results from the yeast two-hybrid screen that identified a truncated XRCC1 protein, ranging from amino acid 107 to 337 of the wild-type protein, this allowed us to identify a minimal region at the N-terminus between amino acids 107 and 170 of XRCC1 as sufficient for binding to E6.

Interference of the E6 proteins with base excision repair

We next sought to determine the biological significance of the XRCC1 E6 interaction by measuring the influence of E6 on the efficiency of XRCC1-dependent DNA SSB. To achieve this we examined whether co-transfection of E6 with human XRCC1 would impact on the ability of the latter protein to correct the methyl methane sulfate (MMS) sensitivity of the XRCC1 mutant CHO cell line EM9. The MMS sensitivity of EM9 has been attributed to a defect in repair of SSBs arising during DNA base excision repair (Caldecott *et al.*, 1994; Taylor *et al.*, 2000). It has been shown previously that the DNA repair-defective

Fig. 5. XRCC1-dependent cell survival MMS assay. EM9 cells (10^3) seeded in each well of a 96-well tissue culture microtitre plate were treated for 1 h with 0.3 mM MMS after transfection with 30 ng of expression vector for human XRCC1 and either 3 µg of empty expression vector pLXSN (2) or 0.3 µg (3), 1 µg (4) or 3 µg (5) of expression vector for HPV16 E6. The total amount of DNA in each transfection was kept constant by equalizing with empty expression vector. After 48 h, cells were incubated with 50 µl of XTT per 100 µl of cell culture medium for 4 h and the spectrophotometric absorbance ($A_{490\text{ nm}}/A_{690\text{ nm}}$) of the cell supernatant, containing a formazan dye formed by mitochondrial dehydrogenases of living cells, was measured in an ELISA reader. The percentage of cell survival after MMS treatment was determined by comparison with cells transfected and treated in the same way, except for the MMS treatment. Each bar represents the average of 16 independent transfection experiments. Standard deviations are given as vertical lines within the bars.

phenotypes of EM9 cells, and other *XRCC1* mutant cell lines, can be overcome by restoring *XRCC1* function through transfection of the pCD2EXH-*XRCC1* expression construct (Caldecott *et al.*, 1995) employed here (Figure 4).

EM9 cells were treated with the methylating agent MMS and surviving colonies were counted 48 h later. The survival rate in MMS-treated, mock-transfected cells was 1.5–2.3% and was increased to 54% ($\pm 13\%$) by transfection of 30 ng of pCD2EXH prior to MMS treatment (data not shown). In each experiment we first transfected EM9 cells with a constant amount of 30 ng of pCD2EXH expression vector together with a constant amount of expression vectors for the different E6 proteins. The survival rates of E6-transfected cells are expressed in relation to survival rates of EM9 cells transfected with pCD2EXH and the empty expression vector plXSN (reference value = 1.0). When we co-transfected initially 1 µg of different E6 expression vectors, we observed a clear decrease in the survival rate by co-transfection with HPV16 E6 (0.6 ± 0.028). Consistent with their lower binding efficiency for XRCC1, as measured by immunoprecipitation analysis, a weaker or lack of effect was observed for HPV1 E6 (0.83 ± 0.65), HPV8 E6 (0.95 ± 0.09) and HPV6 E6 (1.04 ± 0.032 ; data not shown). Given that it gave the greatest reduction in the ability of XRCC1 to facilitate MMS resistance, we concentrated on the HPV16 E6 protein and performed a larger series of experiments in which different XRCC1:E6 ratios were compared for their effect on XRCC1 activity (Figure 4). When using a constant amount of 100 ng of pCD2EXH and increasing amounts of HPV16 E6 expression vector we observed, in eight independent experiments, a clear reduction in the average number of surviving colonies as compared with mock cells transfected with the empty expression vectors pLXSN and pCD2EXH. Transfection with 1 µg of HPV16 E6 yielded,

on average, 42.8 clones after MMS treatment versus 74.7 clones with 1 µg of empty pLXSN expression vector, which is equal to a reduced clonal survival down to 57% due to the activity of E6. Transfection of 3 µg of E6 yielded, on average, 4.76 clones (versus 36.2 clones with pLXSN, equivalent to 13% clonal survival) and 5 µg of E6 led to 20% survival (Figure 4). The decrease observed in levels of overall survival, conferred by increasing amounts of co-transfected vector or E6 expression construct, is most likely due to toxic effects of increasing DNA concentrations in combination with the MMS treatment.

To further examine the impact of E6 on XRCC1 activity, we next established a survival assay that can be evaluated spectrophotometrically. The percentage of cell survival after MMS treatment of E6-transfected cells was determined by comparison with cells transfected and treated in parallel in the same way, except for the MMS treatment. Transfection of *XRCC1* (30 ng of pCD2EXH) into EM9 cells resulted in a 16-fold increase in cell survival (27.7%) after MMS treatment, compared with mock-transfected cell survival (1.75%), which was already significantly reduced by co-transfection with 300 ng of HPV16 E6 to 10.5% (equivalent to a reduced survival rate down to 38%; Figure 5). Although the total levels of cell survival showed a rather high variation between different experiments, the remarkable difference in cell survival between co-transfection with the empty vector plasmid and different amounts of HPV16 E6 expression vector was observed in each experiment.

Discussion

We have shown here that the E6 proteins of the high-risk HPV16, which is present in 50% of all cervical carcinomas worldwide, of HPV8 found in skin carcinomas of patients with epidermodysplasia verruciformis (Orth, 1987; Bosch *et al.*, 1995) and of HPV1, which is the most prevalent type in benign warts, interact with the XRCC1 protein, albeit with different binding affinities. For the E6 protein of HPV6, which is the most prevalent type in benign condylomas, we were not able to detect any interaction with XRCC1, even though we analysed E6 proteins of two different HPV6 subtypes. There is, however, an extremely high variability in the E6 gene of HPV6, with as much as seven unique and eight additional single amino acid exchanges, as found in 16 different clinical isolates (Grassmann *et al.*, 1996), leaving the possibility of an interaction of another HPV6 E6 isolate with XRCC1 still open.

The difference in the binding affinities of the E6 proteins to XRCC1 is reflected in their ability to decrease the DNA repair efficiency in transiently transfected EM9 cells. Whereas the most efficient binding partner, HPV16 E6, was repeatedly able to decrease the XRCC1-mediated MMS resistance to 60%, the E6 protein of HPV1, with 20% binding affinity of HPV16 E6, reduced resistance only to 83% of control levels, and HPV8 E6, with even lower binding affinity, showed no significant decrease in cell survival compared with controls, at least in the transient transfection assay employed here. Although the negative impact of E6 on the activity of XRCC1 in SSBR could easily be explained by an E6-mediated degradation of XRCC1 via the ubiquitin pathway, we found no

evidence for such an activity of E6 (data not shown). However, our experiments that mapped the interacting domain with E6 within XRCC1 to the N-terminus between amino acids 107 and 170, which partially overlaps with the binding domain for DNA polymerase β (Marintchev *et al.*, 1999), open the possibility that the negative effect of E6 on DNA repair could be caused by a displacement of the polymerase from the complex with XRCC1.

Interestingly, an interaction of another human tumour-virus protein, the tax protein of human T-cell leukaemia (HTLV)-1 virus, with a SSBR protein has been reported. Tax interacts with poly (ADP-ribose) polymerase, which is involved in the same repair pathway as XRCC1, and the presence of tax in transfected cells decreased the ability to repair damage induced by UV, quercetin or hydrogen peroxide (Philpott *et al.*, 1999; Anderson *et al.*, 2000). Furthermore, the X-protein of the hepatitis B virus was shown to interact with the UV-damaged DNA-binding protein, although the functional consequence of this interaction is to date unknown (Lee *et al.*, 1995).

The impact of HPV16 E6 on SSBR might be expected to increase genetic instability in cells infected with the virus. Indeed, it has been shown that HPV16-immortalized cells possess genetic instability and increased mutation frequencies (Oda *et al.*, 1996; Shin *et al.*, 1996; Solinas-Toldo *et al.*, 1997), and that cervical intra-epithelial neoplasias and carcinomas frequently harbour chromosomal alterations (Steenbergen *et al.*, 1998; Matthews *et al.*, 2000; Sherwood *et al.*, 2000; Chuaqui *et al.*, 2001), which has so far been attributed to the loss of the p53 protein due to E6-induced degradation (Mantovani and Banks, 2001). Genetic alterations are a constant phenomenon also observed in cancers outside the genital tract, such as in the head and neck and non-melanoma skin cancers, where in addition to HPV16 (Mork *et al.*, 2001) other HPV types, such as HPV1, HPV8 and HPV33, have been found (A.Iftner, S.J.Klug, C.Garbe, A.Blum, A.Stancu, S.P.Wilczynski and T.Iftner, submitted). Mutagenic events may also affect the viral genome, which in the case of the E1 and E2 genes, whose products are involved in viral DNA replication, could lead to the frequently observed integration of HPV DNA into the genome of the persistently infected cell (Cullen *et al.*, 1991).

In addition to the mutagenic activity, which could contribute to the carcinogenic potential of papillomaviruses, one might ask what biological role the interaction of XRCC1 with the E6 protein might fulfil? XRCC1 appears to function as a scaffold protein, recruiting and modulating the enzymatic components of SSBR at sites of DNA damage. One possibility could be that binding of E6 serves to recruit this repair machinery to sites of DNA damage within the viral genome, at a cost to the stability of the host genome. Alternatively, perhaps binding to XRCC1 serves a more direct role in the viral life cycle. For example, one of the binding partners of XRCC1 is DNA polymerase β (Caldecott *et al.*, 1996; Kubota *et al.*, 1996), which can substitute for DNA polymerase α , the principal replicative enzyme driving DNA synthesis of the cellular and viral genome (Müller *et al.*, 1994; Miscia *et al.*, 1997), in cells undergoing apoptosis. A process similar to apoptosis is observed in terminal differentiation seen in superficial cell layers of the epithelium where HPV

DNA amplification during productive infection takes place (Stubenrauch and Laimins, 1999). Our data suggest that the interaction domains of XRCC1 with DNA polymerase β and E6 partially overlap (Marintchev *et al.*, 1999; Figure 3) and that DNA polymerase β might be displaced from XRCC1 by E6. This released enzyme might then play a role in papillomavirus DNA amplification in terminally differentiated keratinocytes. Indirect support for a possible role of E6 in replication comes from studies using viral genomes mutated in the E6 gene that were defective in stable viral DNA replication in transfected primary human keratinocytes (Thomas *et al.*, 1999).

In summary, these data provide an alternative explanation for the genetic instability observed in human tumours persistently infected with papillomaviruses. Besides the established inactivation of p53 by E6, which is only valid so far for a limited number of high-risk genital papillomavirus types, we show that E6 binds and inhibits the DNA strand break repair activity of human XRCC1, a protein known to be required for the maintenance of genetic integrity.

Materials and methods

Plasmid constructions

Plasmids encoding fusion proteins between the DNA binding domain (DBD) of GAL4 (amino acids 1–147) and E6 were constructed by PCR amplification using the primers 5'-GCACAGGACCATATGGCGAC-ACCAATCCGG-3' (*NdeI*) and 5'-CGCCACCGGATCCATATAGCA-TACAAGCGACA-3' (*BamHI*) of the coding sequence for HPV1 E6 from plasmids pBR322/HPV1 (Danos *et al.*, 1983), for HPV16 E6 using the primers 5'-AAGAGAACTGCCATGGTTCAGGACCCA-CAG-3' (*NcoI*) and 5'-CATGCGGATCCACAGCTGGGTTTCTCTACG-TGT-3' (*BamHI*) from pBR322/HPV16 (Seedorf *et al.*, 1985), for HPV8 E6 using the primers 5'-TTTCCTAAGCATATGGACGGGACAGAC-3' (*NdeI*) and 5'-ACAGTGACGGATCCACCAATCATGATACAA-ATG-3' (*BamHI*) from pSVL86/7 (Iftner *et al.*, 1990), and for HPV6 E6 using the primers 5'-GAGGCATTATGGAAAGTGCAAATGCC-3' and 5'-GGAAGACATGTTACCCTAAAGGATATTGT-3' from plasmid HPV6a-pBR322 (de Villiers *et al.*, 1981). All primers contained recognition sequences for restriction enzymes used for cloning that were also present in the GAL4 binding domain yeast expression vector pAS2 used to produce fusion proteins with the GAL4 DBD. PCR-amplified products were cleaved with appropriate restriction enzymes and inserted into the multiple cloning site of the vector pAS2 (Harper *et al.*, 1993). The resulting plasmids encode the GAL4 DBD fused in-frame with the E6 proteins. Constructs based on pAS2 additionally contain a short nucleotide stretch of 27 nucleotides encoding the haemagglutinin (HA) epitope in-frame between the coding sequences of GAL4 and E6. Constructs for *in vitro* transcription/translation were subcloned in pBS⁺ (Stratagene Europe). All cloned amplicates were sequenced following a standard protocol (Ausubel *et al.*, 1990). For prokaryotic expression, HPV16 E6 and HPV6 E2 were cloned into pQE9 (Qiagen, Hilden, The Netherlands) and purified by Ni-NTA technology. To produce soluble HPV16 E6 protein, the E6 gene was cloned into pET33b (Novagen, Germany) and also purified by Ni-NTA technology. Expression vectors for XRCC1 and DNA ligase III have been described previously (Taylor *et al.*, 1988; Caldecott *et al.*, 1992, 1995). Fragments encoding human XRCC1 from amino acids 1–170 (XRCC1_{1–170}) or 402–633 (XRCC1_{402–633}) were cloned into the prokaryotic expression vector pET16b (Novagen) and proteins were purified by Ni-NTA technology.

Construction of a cDNA library from HT3 cells

For construction of a cDNA library, we used the two-hybrid cDNA library construction kit (Clontech, USA) and 10 μ g of poly(A)⁺ RNA isolated from HT3 cells (Nasseri *et al.*, 1982; Chomzynski and Sacchi, 1987). All cDNA fragments were directly cloned into the GAL4 activation domain (AD) yeast expression vector pGAD10 to produce fusion proteins with the GAL4 C-terminal AD. The library consisted of 1.5×10^6 independ-

ent clones and contained cDNA inserts ranging in size between 100 and 4000 bp.

Two-hybrid screen

Y190 yeast cells were co-transformed with 2 µg of pAS2-8E6 as bait, 5 µg of cDNA-pGAD10 and 100 µg of herring sperm DNA using lithium acetate according to Soni *et al.* (1993), and plated onto minimal synthetic media (SD) without leucine and tryptophan (SD-LT) or without leucine, tryptophan and histidine (SD-LTH), containing 7 mM 3-amino-1,2,4-triazole (Sigma, München, Germany) to suppress leaky expression of the *HIS3* gene (Durfee *et al.*, 1993; Harper *et al.*, 1993). For blue/white screening, Leu⁺ and Trp⁺ transformants were incubated for 5 min with chloroform, dried and overlaid with 1% low-melting agarose (FMC Bioproducts, USA) containing 1 mg/ml X-Gal (Roth, Germany) and 100 mM KPO₄, and cooled to 42°C. The plates were incubated at 30°C for 24 h. Colonies that turned blue due to β-galactosidase expression were isolated on a fresh SD-LT plate. Transformation efficiency, estimated by the number of clones on SD-LT, was usually 5 × 10⁴ per µg DNA. Clones growing on SD-LTH that were indicative for protein–protein interaction and tested positive by the β-galactosidase filter assay were retested with the quantitative liquid-phase ONPG test. β-galactosidase units were determined according to Miller (1979) as follows: 1 unit (U) of β-Gal = 1000 × OD_{420nm} / (T × V × OD_{600nm}), where *T* is the time of incubation, *V* is the volume of the starter culture, OD_{420nm} is the absorption of *O*-nitrophenol and OD_{600nm} is the absorption of the starter culture. At least five independent transformants were analysed and each assay was performed several times. To verify positive results from the ONPG assay, positive clones were plated on MM-L that contained in addition cycloheximide (10 µg/ml), which selects for yeast clones that still contain the cDNA-pGAD10 expression vector, but have lost the pAS2-8E6 bait construct. Plasmid DNA was isolated from the resulting yeast clones and transformed in *E. coli* KC8 (leu⁻).

In vitro transcription/translation and co-immunoprecipitation

For the expression of HPV E6 proteins we used T3 or T7 RNA polymerase and a combined *in vitro* transcription/translation system (TNT wheat germ extract; Promega, Germany) according to the manufacturer's instructions. To generate radioactively labelled proteins, translations were performed in the presence of [³⁵S]methionine–cysteine translabel (Amersham, Germany). Total reaction mixtures were then electrophoresed by 15% SDS–PAGE and the radioactively labelled proteins were visualized by fluorography. For co-immunoprecipitation assays, 100 µl extracts from EM9 and EM9-XH cells (Caldecott *et al.*, 1994; Taylor *et al.*, 2000) were mixed with equivalent amounts of *in vitro* translated [³⁵S]methionine–cysteine-labelled E6 proteins and incubated with antiserum at 4°C overnight. Protein A–Sephacryl (30 µl) (50 mg/ml in cell lysis buffer) was added for 1 h at room temperature and after the final wash beads were boiled for 5 min in SDS loading buffer and the proteins were subjected to SDS–PAGE. Co-precipitated E6 proteins were visualized by autoradiography and analysed with the help of a phosphorimager (BAS1800; Fuji Systems, Japan).

Western blotting

For western blotting, yeast clones co-transformed with E6 DBD and the GAL4 AD cDNA library were grown in 5 ml of SD medium and harvested at OD₆₀₀ = 0.7. Cells were quickly chilled on ice, centrifuged in an Eppendorf 5417R centrifuge at 20 000 *g* and resuspended in 500 µl of cold IP buffer (1% Triton X-100, 0.2% SDS, 0.3% deoxycholate, 50 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl pH 8 and protease inhibitors). An equal volume of glass beads was added and cells were lysed with six pulses of vortexing for 30 s separated by 30 s incubations on ice. An additional 500 µl aliquot of IP buffer was added and the extract was clarified by centrifugation in an Eppendorf 5417R centrifuge at 20 000 *g*. Protein extract (100 µl) was mixed with an equal amount of standard sample buffer and proteins were separated by a 15% SDS–PAGE. Western blotting was performed by standard technique, as described previously (Coligan *et al.*, 1999). For detection of the fusion proteins consisting of the GAL4 DBD and E6, a monoclonal antibody specific for HA (HISS Diagnostics, Freiburg, Germany) was used in concentrations as recommended by the manufacturer. For detection of XRCC1, we used the monoclonal antibody mAb33-2-5 (Caldecott *et al.*, 1995). Proteins were visualized by the ECL western blotting system (Amersham), as recommended by the manufacturer.

Far western blotting

To probe the interaction of XRCC1 with immobilized E6 protein, the same amounts of purified HPV16 E6, DNA ligase III and HPV6 E2 proteins were separated by 12% SDS–PAGE (29:1, acrylamide:bisacrylamide) and electrotransferred for 1 h and 90 V at 4°C to nitrocellulose membranes (Amersham). The immobilized proteins were then denatured by incubation (20 min at 4°C) in a buffer containing 6 M guanidine–HCl, 25 mM HEPES–KOH pH 7.7, 25 mM NaCl, 5 mM MgCl₂ and 1 mM DTT. Renaturation was then carried out by successive incubations in the same buffer as above containing decreasing concentrations of guanidine–HCl, the final incubation being carried out in the absence of a denaturing agent. Membranes were then blocked for 1 h at 4°C in 25 mM HEPES–KOH pH 7.7, 25 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.05% NP-40 and 5% milk powder solution, and incubated overnight with XRCC1–His phosphorylated by casein kinase II (Roche Diagnostics, Germany) in the presence of [γ-³²P]ATP (3000 Ci/mmol; Amersham) at 4°C in 5 ml of hybridization buffer (20 mM HEPES–KOH pH 7.7, 0.1 mM EDTA, 2.5 mM MgCl₂, 1 mM DTT, 0.05% NP-40 and 1% milk powder solution). The membranes were washed three times at 4°C with hybridization buffer without the probe, dried briefly and visualized by autoradiography. For probing immobilized XRCC1 mutant proteins with HPV16 E6, equivalent amounts of affinity-purified XRCC1_{402–633} and XRCC1_{1–170} proteins, as estimated from Coomassie Blue-stained gels, were separated by 15% SDS–PAGE. Proteins were transferred to a nitrocellulose membrane (0.2 µm pore size) for 1 h at 70 V with cooling. Membranes were incubated with binding buffer [25 mM HEPES pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.05% (v/v) NP-40 and 5% BSA] for 2 h at 4°C with rocking. The binding buffer was then replaced with 10 ml of fresh buffer containing 1.5% BSA, and ~30 µg of affinity-purified T7-tagged HPV16 E6 protein were added and incubated overnight at 4°C with gentle rocking. After washing with TBS, T7 tag–HRP-conjugated antibody (Novagen 69048) was added at a 1:5000 dilution in binding buffer/1.5% BSA and incubated at 4°C for 1 h. After several washes with TBS/0.05% NP-40, bound antibody was detected by chemiluminescence with SuperSignal West Femto substrate (Perbio Science, Germany). Following that, the blot was washed with TBS and blocked in TBS/0.1% Tween-20/5% skimmed milk powder for 2 h at room temperature. Polyclonal rabbit XRCC1 antiserum (Serotec, UK; AHP 428) was added at a dilution of 1:5000 in TBS/0.1% Tween-20/5% skimmed milk powder and incubated overnight at 4°C with rocking. Then an HRP-conjugated anti-rabbit antibody (diluted 1:3000) was added for 1 h and bound antibody was detected by chemiluminescence after extensive washing with TBS/0.1% Tween-20.

Cell culture, MMS treatment and measurement of cell survival/growth

EM9 cells were grown in α-Medium (Invitrogen, Germany) containing 10% FCS (Seromed Biochrom, Berlin, Germany). Transfections were performed as follows. Lipofectamine (5 µl) (Invitrogen) was diluted in 100 µl of OPTI-MEM I (Invitrogen) and added to a mixture of 100 µl of DNA and OPTI-MEM I, followed by 30 min incubation at room temperature. After the addition of 800 µl of OPTI-MEM, the transfection solution was added to 10 000 EM9 cells seeded in 30 mm cell culture dishes the day before and where the medium was completely removed. Cells were incubated for 5 h, after which 1 ml of alpha-MEM containing 20% FCS was added. After 12 h, the transfected cells were trypsinized and split 1:10 into eight wells of a 24-well cell culture plate and incubated for at least 4 h at 37°C and 7.5% CO₂. Cells were then treated with 0.3 mM MMS (Merck, Darmstadt, Germany) for 1 h or left untreated as mock control. Both treated and control cells were then washed twice with PBS and kept in alpha-MEM containing 10% FCS for 48 h, after which surviving colonies consisting of a minimal 30 cells were counted from four microscopic visual fields. In a second approach, we directly quantified surviving cells with the help of a colorimetric assay (XTT; sodium 3'-[1-(phenyl-amino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) labelling reagent; Roche Diagnostics) as follows: 10³ cells were seeded in each well of a 96-well cell culture plate and transfected as well as MMS treated, as described previously in this paper. The total amount of DNA transfected into each well was kept constant by normalizing with the empty expression vector. After treatment (48 h), the cells were incubated for an additional 4 h in the presence of 50 µl of XTT per 100 µl of cell culture medium and further treated as specified by the manufacturer. Cell survival after MMS treatment of E6-transfected cells was determined by comparison with cells transfected and handled in parallel in the same way, except for the MMS treatment.

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