Activation of protein kinase CK2 is an early step in the ultraviolet B-mediated increase in interstitial collagenase (matrix metalloproteinase-1; MMP-1) and stromelysin-1 (MMP-3) protein levels in human dermal fibroblasts

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Enhanced expression of matrix metalloproteinase (MMP)-1/interstitial collagenase and MMP-3/stromelysin-1 in skin fibroblasts and subsequent damage of dermal connective tissue in the context of sun-induced premature aging and skin tumour progression is causally linked to UVB irradiation. Here, we were interested in identifying components of the complex signal-transduction pathway underlying UVB-mediated up-regulation of these delayed UV-responsive genes and focused on components maximally activated early after irradiation. A 2.3-fold increase in protein kinase CK2 activity was measured at 20–40 min after low-dose UVB irradiation (at 10 mJ/cm²) of dermal fibroblasts. This UVB-mediated increase in CK2 activity was abrogated by pharmacological approaches using non-toxic concentrations of the CK2 inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). Preincubation of fibroblasts with DRB prior to UVB irradiation lowered MMP-1 by 49–69 % and MMP-3 protein levels by 55–63 % compared with UVB-irradiated controls. By contrast, the CK2 inhibitor did not affect the UVB-triggered transcription of MMPs. Furthermore, UVB irradiation of fibroblasts overexpressing a kinase-inactive mutant of CK2 (CK2α-K68A-HA) resulted in lowering of the protein levels of MMP-1 by 25 % and MMP-3 by 22 % compared with irradiated fibroblasts transfected with the vector control. This reduction in MMP protein levels correlated with the transfection efficiency. Taken together, we describe a novel aspect of protein kinase CK2, namely its inducible activity by UVB irradiation, and provide evidence that CK2 is an early mediator of the UVB-dependent up-regulation of MMP-1 and MMP-3 translation, whereas their major tissue inhibitor of matrix metalloproteinase-1 is not affected by CK2.

Key words: photoaging, signal transduction, skin cancer, UVB.

INTRODUCTION

The range of wavelengths of solar radiation that is biologically relevant in physiological and pathophysiological processes is in the UV spectrum, from 280 to 320 nm (UVB) and from 320 to 400 nm (UVA; for review, see [1]). Over several years lowering of the global ozone concentration has been observed, resulting in the thinning of stratospheric ozone and a subsequent increase of UVB doses on the Earth, which is now becoming a major health concern [2,3]. Among the detrimental effects of UV irradiation [4,5] an increase in non-melanoma and melanoma skin cancer [6] as well as premature skin aging (photoaging) [7,8] comprise the most important health hazards. Apart from UVA [9], UVB plays a critical role in photoaging processes [6], which are mediated by both UVB-irradiation-triggered generation of oxidative stress [10,11] and by DNA lesions generated directly by UVB in skin cells [12,13], eventually leading to connective-tissue breakdown and carcinoma formation [1,8,14].

Mammalian cells react to irradiation by UV light with a number of genetic changes, including the expression of distinct genes, which has been named the UV response [15]. UVB-inducible genes comprise several proteinases, among them matrix metalloproteinases (MMPs), a superfamily of zinc-dependent endopeptidases that is capable of degrading all extracellular matrix components [8,16–18]. As to their proteolytic activity, various UVB-induced MMPs in dermal fibroblasts contribute to the breakdown of dermal interstitial collagen and other extracellular matrix components and thus, apart from promoting tumour invasion and metastasis [19], initiate and drive dermal photoaging [6,7,10,20]. Over the past few years, many studies have focused on the complex signalling pathways underlying UVB-mediated up-regulation of delayed UV-responsive genes [21], like interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3), two major contributors to photoaging processes [16,17]. Two signalling pathways for the UV response have been identified. The first pathway originates in the cell nucleus with DNA damage as the primary signal. UV-irradiation-dependent generation of reactive oxygen species near or within the cell membrane is the initiator for the second signalling pathway [22]. Finally, both pathways result, via the involvement of protein kinases and/or phosphatases, in activation of transcription factors leading to increased expression of specific genes. Recently, several components of the stress-activated intracellular signalling pathways were identified to be activated by UVB-generated lipid hydroperoxides and hydroxyl radicals [10] and to be involved in the UVB-triggered induction of MMP-1 and MMP-3 [10,17,20]. In addition, there is independent evidence that both the induction of inflammatory cytokines and the induction of delayed UV-responsive genes, like interstitial collagenase/MMP-1, require UVB- or UVC-damaged DNA as a primary chromophore [12,

Abbreviations used: MMP, matrix metalloproteinase; PI 3-kinase, phosphoinositide 3-kinase; TIMP-1, tissue inhibitor of matrix metalloproteinases-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; HA, haemagglutinin; p70S6k, p70 ribosomal S6 kinase.

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21,23]. In this context, DNA-damage-dependent FK506-binding protein-12/rapamycin-associated protein kinase (FRAP), belonging to the phosphoinositide 3-kinase (PI 3-kinase)-like family [24], and its downstream target p70 ribosomal S6 kinase (p70S6k), have been shown to be critically involved in both the UVB-mediated induction of MMP-1 and MMP-3 in fibroblasts [12] and specific tumour necrosis factor α mRNA level in keratinocyte cell cultures [23]. Besides the causal role of MMPs in degradation of structural proteins like interstitial collagens, the accumulation of collagen degradation products occurring in photodamaged skin has also recently been shown to inhibit type I procollagen synthesis [25].

Several pieces of the puzzle concerning the UVB-initiated activation of signal-transduction pathways, finally leading to the expression of various MMPs, have been found over the past years. However, critical pieces (components) in the induction of delayed UV-responsive genes like MMP-1 and MMP-3 are still missing. In this study, we focused on the identification of UVB-dependent early signalling components that were activated maximally early after the initial UVB irradiation event and which are responsible for MMP-1 and MMP-3 induction. Using a combined pharmacological and genetic approach with CK2 inhibitors and a kinase-inactive CK2 mutant, we have identified that UVB irradiation activates CK2 which is critical for downstream signalling of the UVB response, finally resulting in the induction of two major MMPs. Thus we describe a novel, as yet unreported, role for CK2 that was earlier shown to be essential for proliferation and development in eukaryotes [26].

MATERIALS AND METHODS

Materials

Cell culture medium and TriFast™ reagent were purchased from Life Technologies (Karlsruhe, Germany) and peqlab Biotechnologie GmbH (Erlangen, Germany), respectively, and fetal calf serum (FCS) was from Biochrom (Berlin, Germany). All chemicals and biochemicals were obtained from Sigma (Taufkirchen, Germany) unless otherwise indicated. Transfection reagent Fugene 6 was purchased from Roche Molecular Biochemicals (Mannheim, Germany) and OPTI-MEM I was from Life Technologies. Specific rabbit polyclonal protein kinase CK2 antibody [raised against amino acids 70–89 (LKPVKKKKIKREIKILENLR) of the α-subunit] were provided by Calbiochem (Bad Soden, Germany). Fully active CK2 protein ($\alpha_2\beta_2$), and components of the protein kinase CK2 assay were from Upstate Biotechnology/BIOMOL (Hamburg, Germany). MMP-1, MMP-3 and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) ELISA kits (Biotrak) were obtained from Amersham Bioscience (Braunschweig, Germany). Rabbit polyclonal antibody against human p53 was from Santa Cruz Biotechnology (Heidelberg, Germany), rabbit polyclonal phospho-p53 (Ser-392) antibody and Phototope-HRP Western detection kit were supplied by New England BioLabs (Schwalbach, Germany). Rabbit polyclonal anti-human gelatinase A (MMP-2) and type I collagenase (MMP-1) activity assay kit were obtained from Chemicon International (Hofheim, Germany).

The cDNA clone for TIMP-1 (TIMP p-3.9x; A.T.C.C. code 59666) was provided by the A.T.C.C. (Rockville, MD, U.S.A.). A 920 bp *Hin*dIII/*Sma*I fragment of human interstitial collagenase cDNA (MMP-1) [27] was used as a probe in Northern-blot analysis. The plasmid pSG5 encoding the kinase-inactive mutant of CK2α catalytic subunit (with the substitution Lys-68 → Ala) C-terminally tagged with haemagglutinin (HA) epitope (CK2α-K68A-HA) was a kind gift from Dr E. M. Chambaz and Dr C. Cochet (INSERM, Grenoble, France).

Fibroblast cell cultures

Dermal foreskin fibroblasts were established by outgrowth from biopsies of healthy human donors [28] with an age of 3–6 years and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine (2 mM), penicillin (400 units/ml), streptomycin (50 mg/ml) and 10 % FCS in a humidified atmosphere of 5 % CO_2/95 % air at 37 °C. Cells were used at passages 7–18, corresponding to cumulative population-doubling levels of 13–37 [29]. Confluent and serum-starved (0.2 % FCS, 24 h) cells were preincubated for 1 h with non-toxic concentrations of the PI 3-kinase and CK2 inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; 5 or 10 μM] [30] or the specific PI 3-kinase inhibitor wortmannin (0.1 or 0.3 μM) [30] or for 16 h with the specific CK2 inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; 1–10 μM) [31]. Subsequently, the fibroblast monolayer cultures were irradiated with the total UVB spectrum (280–320 nm) at a dose of 10 mJ/cm² as described elsewhere [12].

Light source and UV irradiation

A 1000 W xenon high-pressure UV source was used in conjunction with a monochromator with holographic grating (Dermolum UMW; Fa. Müller, Moosinning, Germany). For the experiments, the total UVB spectrum (280–320 nm) was used. Fluence rates were determined by an integrated thermopile. The dose rates on the cell surface were 0.48 mW/cm² for the total UVB spectrum. To guarantee a constant intensity and spectral distribution, the spectral distribution was measured by spectroradiometry with an OL-754 UV/visible light spectroradiometer (Optronic, Orlando, FL, U.S.A.). Confluent fibroblast monolayer cultures were rinsed twice with PBS, and all irradiations were performed under a thin layer of PBS. There was negligible loss in the viability of cell populations compared with cells held under similar conditions without irradiation [16]. Following UVB irradiation, cells were washed with PBS and cultured in FCS-free DMEM with or without wortmannin, LY294002 or DRB for various periods of time, dependent on the experimental settings.

Cytotoxicity assay and viability

The viability of confluent dermal fibroblasts was measured 24 h after incubation with the protein kinase inhibitors wortmannin, LY294002 and DRB. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was used for the quantification of living metabolically active cells as described elsewhere [32] with minor modifications. Mitochondrial dehydrogenases metabolize MTT to purple formazan dye during an incubation time of 1 h at 37 °C and at a concentration of 0.5 mg of MTT/ml of medium. Thereafter, the cells were lysed with 2 ml of 100 % DMSO per tissue-culture dish (diameter, 3.5 cm). Lysates were measured spectrophotometrically at 550 nm. Cytotoxicity was calculated as the percentage of formazan formation in cells treated with the agents compared with mock-treated cells.

Measurements of MMP protein levels: ELISA

MMP-1, MMP-3 and TIMP-1 'sandwich' ELISA assays were performed according to the manufacturer's protocols (Amersham Bioscience) using precoated 96-well immunoplates, rabbit anti-human MMP-1, MMP-3 or TIMP-1 antibodies, and anti-rabbit horseradish peroxidase conjugate. 3,3′,5,5′-Tetramethylbenzidine ('TMB') was used as a peroxidase substrate. Absorbances were read at 450 nm using a microtitre plate reader LP400 (Sanofi Diagnostics Pasteur, Freiburg, Germany). Concentra-

tions of MMP-1, MMP-3 and TIMP-1 in the samples were determined against standard curves using GraphPad™ software (San Diego, CA, U.S.A.).

RNA extraction and Northern-blot analysis

Total RNA was isolated and subjected to Northern-blot analysis and hybridized sequentially with specific cDNA probes for interstitial collagenase (MMP-1) and TIMP-1, as published elsewhere [16]. Briefly, after preparation of total RNA from fibroblasts using the TriFast™ method, equal amounts (5 μg/lane) were fractionated by size on a 0.8 % 2.2 M formaldehyde/agarose gel. After diffusion transfer to nitrocellulose filters using 20 × SSC (3.0 M NaCl/0.3 M sodium citrate, pH 7.0) and vacuum baking (Schleicher & Schuell, Dassel, Germany), hybridizations were performed using denatured α-^{32}P-labelled cDNA probes generated by random-prime technology as described by the manufacturer (Megaprime DNA Labelling System; Amersham Bioscience). Densitometric analysis was performed using the ScanPackII system (Biometra, Göttingen, Germany).

Interstitial collagenase/MMP-1 activity assay

The MMP-1 activity assay, which is based upon fluorescence measurement of collagen fragments upon cleavage by MMP-1, was performed according to the manufacturer's protocol. Upon MMP-1 cleavage of fluorescently FITC-labelled collagen type I, decomposition fragments were produced that were selectively denatured and extracted with ethanol. The fluorescence intensity of the extracted product was measured at 520 nm (emission) and 495 nm (excitation) and the MMP-1 activity (units/ml) calculated as described above. To avoid artificial results due to many proteinases exhibiting collagen-decomposing activity, the serine proteinase inhibitor PMSF was added to supernatants at a final concentration of 1 mM. Although MMP-2 and MMP-13 are not inducible by UVB [17,33], MMP-2 was removed from cell-culture supernatants by immunoprecipitation with a polyclonal antibody (1:500 diluted in supernatant), as described below, prior to MMP-1 activity measurements.

Immunoprecipitation and protein kinase CK2 assay

The activity of the kinase CK2 was determined by incorporation of ^{32}P into synthetic peptide RRRDDDSDDD as described previously [34] with minor modifications. Briefly, after a 24 h starvation period (0.2 % FCS in DMEM), confluent fibroblasts were irradiated at a dose of 10 mJ/cm^2 UVB. At different time points thereafter, cells were rinsed twice with ice-cold PBS, then lysed in ice-cold lysis buffer (50 mM Hepes, pH 7.4, 1 % Nonidet P-40, 100 mM NaCl, 1 mM EGTA, 1 mM PMSF, 10 μg/ml of each of aprotinin, leupeptin, and pepstatin and 1 mM activated Na_3VO_4). Cell lysates were precleared (100 μl of Protein A–agarose bead slurry/ml of lysate) for 1 h at 4 °C and thereafter 500 μl of that lysate (1 μg of total protein/μl of buffer) were incubated with 3–4 μg of a specific rabbit polyclonal antibody raised against amino acids 70–89 of the human CK2α subunit at 4 °C for 2 h. The immune complex was absorbed to Protein A–agarose (50 μl of bead slurry) for 1 h at 4 °C and rinsed twice with ice-cold PBS and once with assay dilution buffer (ADB; 20 mM morpholinopropanesulphonic acid, pH 7.2, 25 μM β-glycerol phosphate, 5 mM EGTA, 1 mM Na_3VO_4 and 1 mM dithiothreitol). Half the volume of the resulting precipitate was suspended in 50 μl of reaction volume composed of 10 μl each of 250 mM substrate peptide in ADB, inhibitor cocktail (2 μM protein kinase A inhibitor peptide in ADB) and a magnesium/ATP cocktail {10 μCi [γ-^{32}P]ATP (≈300 Ci/mmol) in 75 mM $MgCl_2$/500 μM ATP}, and incubated for 15 min at 30 °C with gentle agitation. After centrifugation, 25 μl aliquots of supernatant were spotted on to Whatman P81 phosphocellulose paper, and washed five times for 5 min with 0.75 % phosphoric acid and finally twice for 2 min with 96 % ethanol. Radioactivity bound to filters was counted with a scintillation counter. Activity of CK2 per sample was determined as pmol of phosphate incorporated into substrate/min per mg of total protein based on the specific activity of the magnesium/ATP cocktail. As a positive control 25–50 ng of active CK2 (specific activity, 1600 units/mg) were added to the cell lysate before immunoprecipitation.

Western-blot analysis

Western-blot analysis was performed according to standard protocols published elsewhere [35] with minor modifications. Briefly, confluent and serum-starved cells were subjected to specific treatment and cell lysates were prepared as described above. Equal amounts of proteins (50 μg/lane) were resolved by SDS/PAGE (10 % gels) and transferred to nitrocellulose filters. The filters were incubated with a 1:1000 dilution of primary antibodies [rabbit polyclonal protein kinase CK2 antibody, or rabbit polyclonal p53 or phospho-p53 (Ser-392) antibodies] and a 1:1000 dilution of anti-rabbit secondary antibody conjugated with horseradish peroxidase. Antigen–antibody complexes were visualized by enhanced chemiluminescence (Phototope detection kit) and exposed to Kodak X-OMAT AR film. Equivalent loading of proteins in each lane was ascertained by Ponceau S staining of the filters.

Transient transfection

The plasmid pSG5 encoding the kinase-inactive mutant of CK2α catalytic subunit C-terminally tagged with HA epitope (CK2α-K68A-HA) has been described previously [36,37]. pSG5 minus the CK2α insert served as a negative vector control in transfection experiments. The plasmids were co-transfected at a ratio of 10:1 with the β-galactosidase-gene-containing pCMV-βGal vector (Clontech, Heidelberg, Germany) into 80 % confluent human dermal fibroblasts using the Fugene 6 reagent, a blend of lipids and other components, as per the manufacturer's instructions with minor modifications. Briefly, Fugene 6 reagent was diluted directly in FCS-free OPTI-MEM medium and incubated at room temperature for 5 min before the diluted reagent was added to the plasmid DNA in polystyrene tubes. The DNA–Fugene 6 mixtures in the ratio of Fugene 6 volume/DNA amount indicated in Figure 7 (see below) were then incubated at room temperature for an additional 15 min to allow complex formation. The complex (100 μl/3.5 cm-diameter tissue-culture dish) was added to the cells preincubated for 1 h in OPTI-MEM/5 % FCS to get a final volume of 1.5 ml. Transfection mixture was replaced by DMEM/0.5 % FCS after 8 h and the cells were incubated for an additional 16 h prior to UVB irradiation, as indicated above. Then, 24 h after irradiation the supernatants were collected and subjected to MMP detection, and cell lysates were prepared for measurements of β-galactosidase activity to standardize transfection efficiency. Chemiluminescence generated by β-galactosidase activity was measured as described elsewhere [38] using the Galacto-Light™ kit (Tropix, Bedford, MA, U.S.A.).

Staining of β-galactosidase: transfection efficiency

After 48 h of co-transfection with β-galactosidase fibroblasts were fixed with 0.5 % (v/v) glutaraldehyde in PBS for 5 min at

room temperature and thereafter stained overnight at 37 °C with the chromogenic substrate 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside (X-Gal; 1 mg/ml X-Gal in 20 mM potassium ferrocyanide, 20 mM ferricyanide and 10 mM $MgCl_2$). β-Galactosidase converts X-Gal into a blue precipitate, thus allowing the detection of transfected cells [39]. Expression of the *lacZ* gene was considered positive only when there was dark blue staining of the cells. Percentage transfection efficiency was calculated as the ratio of the number of blue cells to the total number of cells.

RESULTS

UVB irradiation leads to Ser-392 phosphorylation of protein p53

Confluent and serum-deprived dermal fibroblasts were irradiated at a dose of 10 mJ/cm² UVB and subjected to Western-blot analysis at different time points after irradiation. A phospho-specific antibody exclusively recognizing phosphorylated tumour-suppressor protein p53 at Ser-392 and a phosphorylation-state-independent p53 antibody were used. Following UVB irradiation, a time-dependent increase in phosphorylation of Ser-392, a specific binding site for protein kinase CK2 [31,40], was observed that peaked at 40–60 min of irradiation with an up to 2.8-fold increase compared with mock-treated cells (C; Figure 1). UVC-irradiated cells, used as a positive control [41], showed a 1.8 ± 0.2-fold increase in Ser-392 phosphorylation at 60 min of irradiation compared with mock-irradiated controls. This increase in Ser-392 phosphorylation rate decreased nearly to the basal level after 120 min of UVB irradiation (results not shown). Conclusively, these data provide the first evidence for a potential involvement of protein kinase CK2 in the UVB response of dermal fibroblasts.

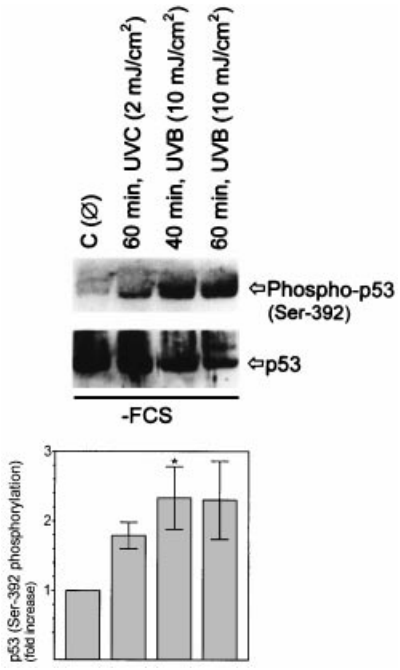

Figure 1 UVB-mediated Ser-392 phosphorylation of tumour-suppressor protein p53

Confluent and serum-starved fibroblast monolayer cultures were irradiated with UVB at a dose of 10 mJ/cm². Using polyclonal antibodies raised against human p53 or phosphorylated Ser-392 of p53, Western-blot analysis was performed as described in the Materials and methods section. Three independent experiments were performed. $^*P = 0.007$ compared with mock-irradiated controls (C; two-tailed Student's *t* test).

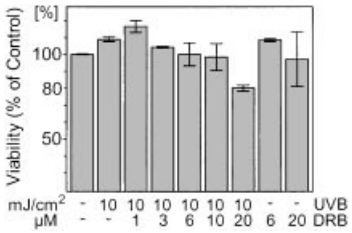

Figure 2 Cytotoxicity of the protein kinase CK2 inhibitor DRB

Confluent mitotic human dermal fibroblasts were pretreated with different concentrations of DRB prior to UVB irradiation at a dose of 10 mJ/cm². The percentage of living cells after an incubation time of 24 h in DMEM without FCS was measured. The data were obtained by four independent experiments.

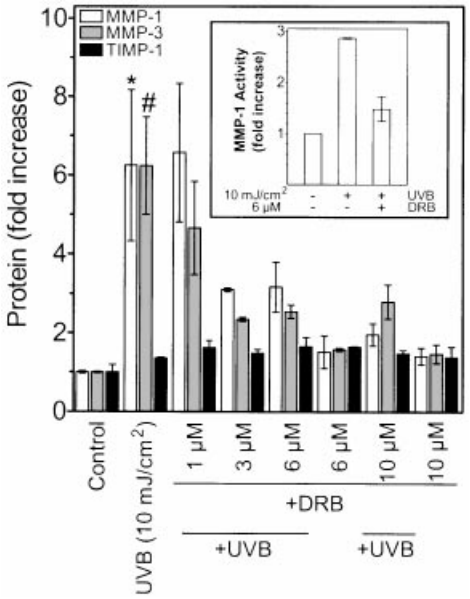

Figure 3 Concentration-dependent effect of protein kinase CK2 inhibitor DRB on UVB-induced MMP-1 and MMP-3 protein levels

Confluent fibroblasts were pretreated or mock-treated with non-toxic concentrations of DRB prior to irradiation with the complete UVB spectrum at a dose of 10 mJ/cm². Supernatants were collected 24 h post-irradiation and subjected to ELISA detection of MMP-1 (white bars), MMP-3 (grey bars) and TIMP-1 (black bars). Cell lysates were used for measurements of MMP-1 activity level (inset). Data represent fold increases in MMP-1, MMP-3 and TIMP-1 protein levels compared with mock-treated controls, which were set at 1.0. Three independent experiments were performed to determine specific proteins. $^*P = 0.0048$ and $\#P = 0.0003$ compared with DRB-treated/UVB-irradiated cells (ANOVA, Dunnett test).

Cytotoxicity of the protein kinase CK2 inhibitor DRB

We intended to avoid interference of cytotoxicity from chemical agents. Therefore, MTT assays were performed to determine optimal non-toxic concentrations at which $\geqslant 80\,\%$ of the mitotic fibroblasts survived at least 24 h after incubation with DRB, with no change in morphology. The maximal concentrations, which fit the above-mentioned requirement, were 20 μM for DRB (Figure 2). For subsequent experiments lower concentrations of maximal 10 μM DRB were applied since combinations of the agent at these concentrations plus UVB irradiation maintained viability between 90 and 100 %. Combinations of the chemical compounds at higher concentrations plus UVB irradiation lowered viability below 80 % (Figure 2). Furthermore, maximal

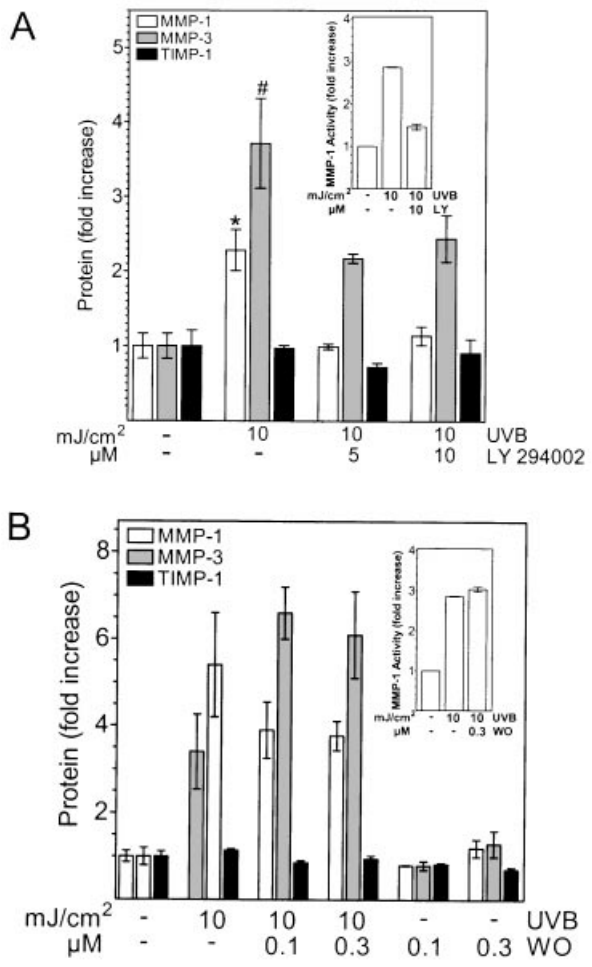

Figure 4 Modulation of UVB-mediated MMP-1 and MMP-3 protein levels by PI 3-kinase, CK2 inhibitor LY294002 and specific PI 3-kinase inhibitor wortmannin (WO)

Confluent fibroblasts were preincubated or mock-incubated with non-toxic concentrations of LY294002 (**A**) or wortmannin (**B**) prior to irradiation at a dose of 10 mJ/cm^2 UVB. Thereafter (24 h), supernatants were collected and subjected to ELISAs for MMP-1 (white bars), MMP-3 (grey bars) and TIMP-1 (black bars). Cell lysates were used for measurements of MMP-1 activity level (inset; units shown are fold increase in activity over the respective controls). Data represent fold increases in MMP-1, MMP-3 and TIMP-1 protein levels compared with mock-treated controls, which were set at 1.0. Experiments were performed in triplicate. (**A**) $*P = 0.0003$ and $\#P = 0.0062$ compared with LY294002-treated/UVB-irradiated cells (ANOVA, Dunnett test).

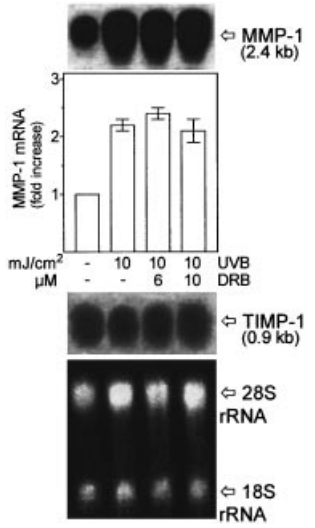

Figure 5 Effect of CK2 inhibitor DRB on UVB-triggered MMP-1 and TIMP-1 steady-state mRNA levels

After preincubation of confluent fibroblast monolayer cultures with non-toxic concentrations of DRB, fibroblasts were irradiated with UVB at a dose of 10 mJ/cm^2. Post-irradiation (24 h), total RNA was isolated and subjected to Northern-blot analysis for the detection of specific MMP-1 and TIMP-1 mRNA levels. Densitometric data were standardized to 18 S rRNA and represent fold increase over the control, which was set at 1.0. A representative Northern blot of three independent experiments is shown.

concentrations of 10 μM for the PI 3-kinase and CK2 inhibitor LY294002 and 0.3 μM for the specific PI3-kinase inhibitor wortmannin were determined which fit the criterion mentioned above (i.e. non-toxicity; results not shown).

UVB-mediated increase in MMP-1 protein and activity level and MMP-3 protein level can be significantly diminished by the CK2 inhibitors DRB and LY294002

In order to study the involvement of protein kinase CK2 in UVB-mediated induction of MMP-1 and MMP-3, we used the specific CK2 inhibitor DRB and LY294002, described recently to inhibit CK2 with a potency similar to that for PI 3-kinase [30]. Additionally, the specific PI 3-kinase inhibitor wortmannin was used to assess the results of LY294002 effects regarding PI 3-kinase involvement in UVB-mediated MMP expression. Confluent fibroblast monolayer cultures were incubated with non-toxic concentrations of DRB (Figure 3), LY294002 (Figure 4A) or wortmannin (Figure 4B) prior to UVB irradiation at a dose of 10 mJ/cm^2. Cell-culture supernatants were subjected to ELISA analysis 24 h post-irradiation (Figures 3 and 4). UVB irradiation resulted in a 6.2-fold increase in both MMP-1 and MMP-3 protein levels compared with mock-irradiated cells. Preincubation of fibroblasts with DRB prior to UVB irradiation lowered MMP-1 protein levels by 49–69 % and MMP-3 protein levels by 55–63 % compared with UVB-irradiated controls (Figure 3). By contrast, TIMP-1 protein levels were almost unchanged under these experimental conditions. The inset in Figure 3 reflects the correlation between UVB-dependent increase in protein levels and enzyme activity. The MMP-1 activity level increased 2.9-fold upon UVB irradiation compared with mock-irradiated fibroblasts. Incubation of the cells with DRB prior to irradiation reduced the activity level by 48 % (Figure 3).

The results obtained by use of CK2 inhibitor DRB were confirmed by the inhibitor LY294002. A 2.3-fold increase in MMP-1 and a 3.7-fold increase in MMP-3 protein levels upon UVB irradiation at 10 mJ/cm^2 compared with mock-treated controls were significantly modulated by preincubation of the cells with non-toxic concentrations of LY294002 (Figure 4A). MMP-1 protein levels were lowered by 91–100 % and MMP-3 levels by 35–40 % compared with UVB-irradiated controls. Furthermore, a 2.9-fold increase in the MMP-1 activity level of irradiated fibroblasts was significantly diminished by 86 % on preincubation with LY294002 (Figure 4A, inset). By contrast, the specific PI 3-kinase inhibitor wortmannin did not show any effect on MMP protein and activity levels. A 3.4-fold increase in MMP-1 and 5.4-fold increase in MMP-3 protein amounts measured in cell-culture supernatants upon UVB irradiation was not modulated by application of non-toxic concentrations of

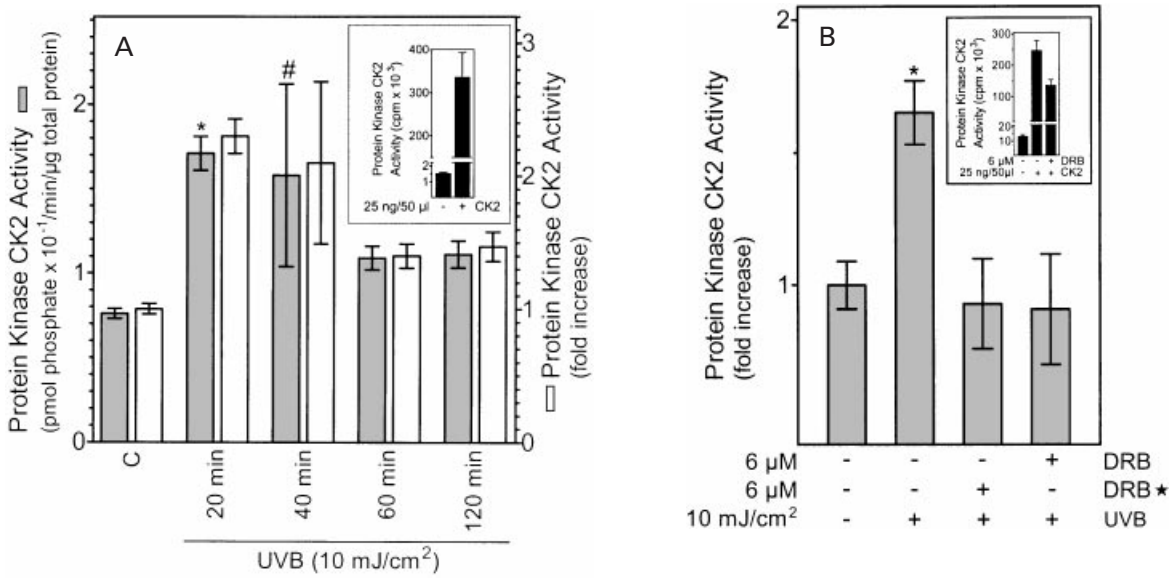

Figure 6 UVB-modulated protein kinase CK2 activity and effect of CK2 inhibitor DRB

Left-hand panel: confluent and serum-starved fibroblasts were subjected to UVB irradiation and CK2 activity was determined at the indicated time points post-irradiation according to the Materials and methods section. The inset in the left-hand panel represents activity of exogeneously added CK2, used as positive assay control, to mock-irradiated controls (given in c.p.m. $\times 10^3$). Right-hand panel: in a subsequent set of experiments confluent, serum-starved fibroblasts were treated or mock-treated with 6 μM DRB prior to UVB irradiation at a dose of 10 mJ/cm^2. Post-irradiation (20 min) CK2 activity was determined as detailed in the Materials and methods section. The inset in the right-hand panel shows that DRB reduces the activity of exogenously added CK2 to cell lysate of mock-irradiated cells (units are c.p.m. $\times 10^3$). The treatment marked * demonstrates the effect of DRB added to cell lysate of irradiated cells. Three independent experiments were performed. Left-hand panel, $^*P < 0.0001$ and $\# P = 0.036$ compared with mock-irradiated controls (C; two-tailed Student's t test). Right-hand panel, $^*P = 0.0028$ compared with DRB-treated/UVB-irradiated cells (ANOVA, Dunnett test).

wortmannin (Figure 4B). These data provide further evidence that CK2 is involved in UVB-triggered up-regulation of MMP-1 and MMP-3 protein levels.

DRB does not exert any effect on UVB-dependent increase in interstitial collagenase (MMP-1) mRNA levels

In order to study whether DRB exerts its effect on the level of transcription, confluent fibroblast monolayer cultures were pre-incubated with the above-mentioned agent prior to UVB irradiation. Thereafter, the cells were subjected to Northern-blot analysis to determine specific mRNA levels of MMP-1 and the tissue inhibitor TIMP-1. Low constitutive levels of MMP-1 and TIMP-1 mRNA were detected in mock-irradiated control cells (Figure 5). UVB irradiation at a dose of 10 mJ/cm^2 resulted in a 2.3-fold increase in MMP-1 steady-state mRNA levels compared with mock-irradiated fibroblasts, whereas TIMP-1 mRNA levels remained unchanged under these experimental conditions. Interestingly, non-toxic concentrations of DRB did not reveal any significant effect on the UVB-induced MMP-1 and TIMP-1 mRNA levels (Figure 5), suggesting that upon UVB-mediated increase in CK2 activity CK2 preferentially affects the translational level of MMP-1 induction.

Diminished UVB-mediated increase in protein kinase CK2 activity by DRB

To address the question of whether protein kinase CK2 is involved directly in the increase in MMP-1 and MMP-3 protein levels upon UVB irradiation, confluent serum-starved fibroblasts were irradiated and subjected to CK2 activity assays at different time points thereafter (Figure 6, left-hand panel). Furthermore, we studied the effect of the CK2 inhibitor DRB on UVB-mediated activity of CK2 (Figure 6, right-hand panel).

Constitutive activity of protein kinase CK2 was detected in mock-irradiated control cells. Following UVB irradiation, a time-dependent increase in CK2 activity was observed which peaked at 20–40 min post-irradiation with a 2.3-fold increase compared with mock-treated controls. This activity decreased 60–120 min post-irradiation without reaching basal levels of mock-treated controls (Figure 6, left-hand panel). The inset (Figure 6, left-hand panel) shows the activity of exogeneously added active protein kinase CK2, used as a positive control, to the cell lysate of non-irradiated fibroblasts, which resulted in a 110-fold higher CK2 activity compared with the activity of endogeneous CK2 of irradiated cells.

Following pretreatment with CK2 inhibitor DRB and subsequent UVB irradiation at a dose of 10 mJ/cm^2, fibroblasts were subjected to CK2 assays at 20 min post-irradiation (Figure 6, right-hand panel). Again, UVB-irradiated fibroblasts showed a significantly induced CK2 activity with a 1.7-fold increase in CK2 activity after UVB irradiation. Interestingly, preincubation of the cells with DRB prior to UVB irradiation or adding DRB to cell lysates of irradiated cells resulted in abrogation of UVB-mediated CK2 activity (Figure 6, right-hand panel). The inset (Figure 6, right-hand panel) shows conclusively that DRB had a significant inhibitory effect on the activity of exogenously added active CK2 to cell lysates of non-irradiated cells.

Overexpression of CK2 kinase-inactive mutant suppresses UVB-induced increase in MMP-1 and MMP-3 protein levels

To further prove the involvement of protein kinase CK2 in UVB-triggered up-regulation of MMP-1 and MMP-3 translation apart

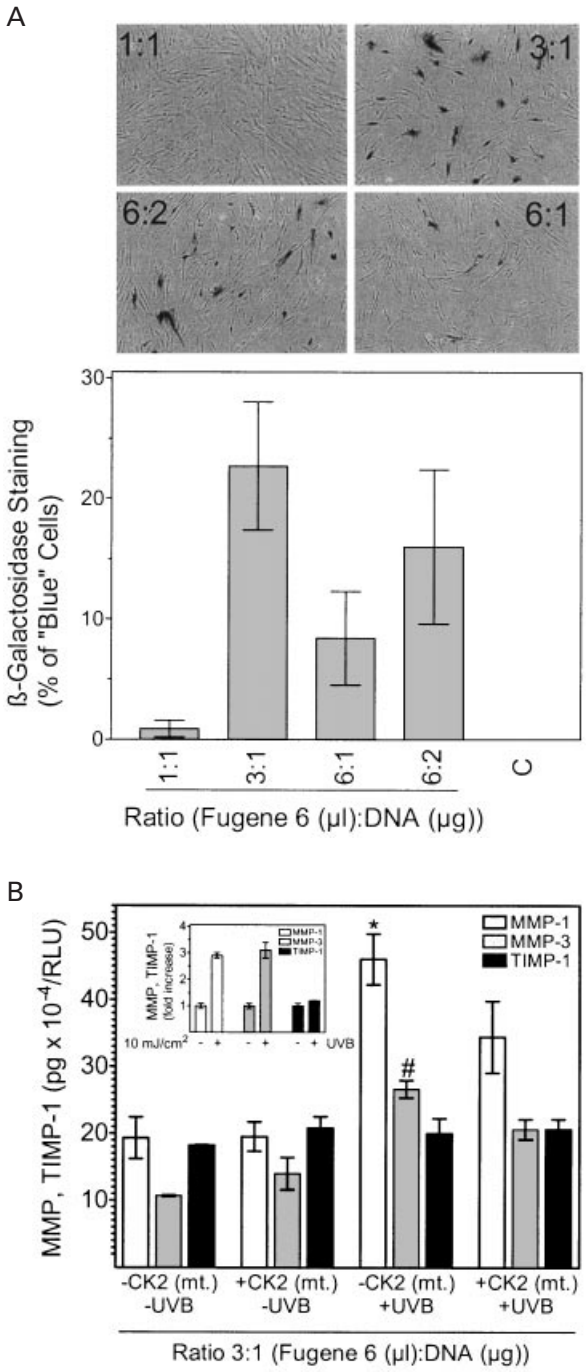

Figure 7 CK2 kinase-inactive mutant-affected expression of MMP-1 and MMP-3 of transfected dermal fibroblasts

(**A**) Subconfluent dermal fibroblasts were transiently co-transfected with a kinase-inactive mutant of CK2α catalytic subunit (CK2α-K68A-HA) or with the plasmid pSG5 alone in combination with a β-galactosidase-containing vector and transfection efficiencies measured as described in the Materials and methods section. (**B**) supernatants were collected 24 h after irradiation at a dose of 10 mJ/cm^2 UVB and subjected to ELISA detection of MMP-1 (white bars), MMP-3 (grey bars) and TIMP-1 (black bars). In parallel, non-transfected fibroblasts used at the same population-doubling level as the transfected cells were UVB-irradiated and tested positively for MMP expression (inset). Three independent experiments were performed to determine specific protein levels. (**A**) C, 1 μg of plasmid DNA without Fugene 6 used as negative control; dark-coloured cells, transfected fibroblasts. (**B**) $-$CK2 (mt), plasmid pSG5 without kinase-inactive CK2 insert; $+$CK2 (mt), CK2α-K68A-HA construct; $-$UVB, mock-irradiated fibroblasts; $+$UVB, UVB-irradiated fibroblasts (dose, 10 mJ/cm^2). $^*P = 0.039$ and $\#P = 0.007$ compared with UVB-irradiated/CK2α-K68A-HA-transfected fibroblasts [$+$CK2 (mt) $+$ UVB; two-tailed Student's t test].

from using a pharmacological approach, human dermal fibroblasts were transiently transfected with a kinase-inactive mutant of CK2α catalytic subunit (CK2α-K68A-HA) [37] or with the ampicillin-resistance plasmid pSG5 alone. The highest transfection efficiency was obtained at a ratio of tranfection reagent and plasmid DNA of 3:1 (Figure 7A). At a ratio of 3:1 a transfection efficiency of $22 \pm 5\%$ was determined, whereas ratios of 6:2 and 6:1 resulted in transfection efficiencies of 16 ± 6 and $8.5 \pm 4\%$, respectively. Therefore, subsequent experiments were performed at a ratio of 3:1. According to the manufacturer's instructions a ratio of 1:1 resulted in less than 1% transfection efficiency. Plasmid DNA without transfection reagent, used as a negative control, was added to the cells and was not incorporated by the fibroblasts (Figure 7A).

Transiently transfected fibroblasts were exposed to UVB irradiation at a dose of 10 mJ/cm^2. Supernatants were collected and subjected to ELISA analysis for MMP-1, MMP-3 and TIMP-1 24 h post-irradiation (Figure 7B). Transfected, irradiated fibroblasts showed 2.4-fold and 2.5-fold increases in MMP-1 and MMP-3 protein levels, respectively, compared with transiently transfected, mock-irradiated cells. By contrast, UVB irradiation of transfected fibroblasts overexpressing the kinase-inactive mutant resulted in reduction of MMP-1 protein level by 25% and of MMP-3 protein level by 22% compared with irradiated fibroblasts transfected with the vector control. By contrast, TIMP-1 protein level of transfected fibroblasts was not affected by UVB irradiation (Figure 7B). The reduction in protein concentration of the specific MMPs correlated well with corresponding transfection efficiencies (Figure 7A). The inset (Figure 7B) shows an increase in MMP-1 and MMP-3 protein levels of non-transfected fibroblasts used as controls at the same cumulative population-doubling level as the transfected cells. Interestingly, the non-transfected and UVB-irradiated cells exhibited higher MMP protein levels than the transfected and UVB-irradiated counterparts, measured as fold increase over controls, which were set at 1.0. The higher MMP protein levels of non-transfected cells (2.9-fold for MMP-1 and 3.1-fold for MMP-3) in comparison with transfected cells (2.4-fold for MMP-1 and 2.5-fold for MMP-3) was due to lower base levels of MMP-1 and MMP-3 protein in non-transfected cells (70.9 ± 4.5 ng of MMP-1/mg of total protein and 46.6 ± 2.6 ng of MMP-3/mg of total protein) versus transfected cells (115.8 ± 18.7 ng of MMP-1/mg of total protein and 64.5 ± 5.8 ng of MMP-3/mg of total protein). We may speculate that the transfection procedure exerted stress on to the cells, finally resulting in an increase in basal MMP protein levels known to be sensitive to stress signals.

DISCUSSION

In this study we have focused on identification of UVB-dependent components of the signal-transduction pathway(s) that (i) causally contribute to the induction of two major members of the MMP family, the interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3), and (ii) were maximally activated early after irradiation. Earlier published data [10,12,20,22,23] led to the conclusion that several pieces of the puzzle concerning UVB-triggered activation of signal-transduction components involved in expression of MMPs are still missing. Herein, we identified protein kinase CK2 to be an early critical component in the UVB-initiated signal cascade(s) leading to an imbalance of proteolysis with an increase in MMP-1 and MMP-3 protein levels and unchanged TIMP-1 protein level, a prerequisite for premature skin aging. This finding is of particular importance in

view of future development of specific antagonists interfering with connective-tissue degradation in skin aging and tumour progression.

DNA-damaging agents such as UV radiation result in activation of tumour-suppressor protein p53, which is reflected by phosphorylation at Ser-15 and Ser-392. Phosphorylation at Ser-392 induces the DNA-binding function of p53 whereas phosphorylation at Ser-15 prevents the binding of MDM2 (the human homologue of mouse double minute-2), a negative regulator of p53. Earlier reports revealed this specific phosphorylation of p53 following UVC irradiation [31,42], which is biologically not relevant as it does not reach the Earth's surface [2]. Using low doses of the complete UVB spectrum, we herein demonstrated a phosphorylation of Ser-392, whereas phosphorylation of Ser-15 could not be detected at the studied time points (results not shown). As Ser-392 is a specific protein kinase CK2 site [31], we addressed the question of whether CK2 plays a role in UVB-mediated induction of MMPs, apart from its role in the activation of p53.

The serine/threonine protein kinase CK2, composed of two catalytic (α and α') and two regulatory (β) subunits, is distributed ubiquitously among eukaryotic cells. Although the precise role of CK2 in cellular responses is poorly understood, CK2 has been reported to be implicated in various steps of development, differentiation, formation of cellular shape and architecture, growth regulation and transformation [26,43]. For example, the average CK2 activity was significantly enhanced in kidney tumours [44] and in metastatic melanoma [45]. Furthermore, a requirement of CK2 for entry into and progress through early phases of the cell cycle [46] as well as for mitosis of fibroblasts [47] could be shown along with its activation by serum, growth factors, hormones, phorbol esters, antibiotics and arsenite [40]. Our major finding is that a physical stimulus, namely UVB radiation at physiological doses, resulted in a significant increase in protein kinase CK2 activity, which, preceding the down-regulation of specific MMP-1 and MMP-3 protein levels, was almost completely abrogated by the CK2 inhibitor DRB. Furthermore, these data suggest a novel role for CK2 to be an essential regulator in the UVB-mediated signalling response leading to induction of distinct MMPs.

In our study a peak of UVB-triggered CK2 activity was measured as early as 20 min after UVB irradiation, suggesting that CK2 belongs to a group of immediate regulators of the UVB response comparable with epidermal growth factor receptor and the GTP-binding regulatory protein Ras, which, similar to CK2, become activated within 30 min of UVB irradiation *in vivo* [20]. By contrast, serum treatment of quiescent NIH 3T3 fibroblasts increased CK2 expression and activity, which peaked at 4 h after treatment [48]. It is possible that time-dependent differences in expression and activity of CK2 and other kinases depend on the stimuli and cell types used for the studies.

The protein kinase CK2 inhibitor DRB has generally been considered to be an indirect RNA polymerase II inhibitor, an effect which seemed to be mediated through inhibition of CK2 lying upstream of the polymerase and consequentially resulting in transcriptional inhibition of most genes [49]. In contrast to its suppressing effects on MMP-1 protein level, the CK2 inhibitor could not prevent the increase in MMP-1 mRNA level throughout the experiments, suggesting a specific regulatory role of CK2 in the translation of the herein-studied MMPs. Hence we identified a novel effector function of CK2 that eventually contributes to connective-tissue degradation. Indeed, UVB irradiation of human skin *in vivo* resulted in enhanced proteolysis of the preferential substrate for MMP-1, interstitial collagens [20].

The finding that CK2 exerts its effect on UVB-triggered up-regulation of distinct MMPs via a suggested regulatory role in their translation took into consideration a possible interaction of CK2 with p70S6k. Recently, p70S6k was shown to have a specific regulatory role in the translation of MMPs after UVB irradiation of human dermal fibroblasts, without any impact on MMP mRNA levels [12]. Furthermore, apart from the finding that DRB blocked insulin-stimulated activity of nuclear and cytosolic p70S6k in Chinese hamster ovary cells [50], it could be demonstrated that nuclear-localization sequences of p70S6k protein revealed a CK2 consensus recognition site [51], resulting in phosphorylation of p70S6k by CK2 [52]. These findings strengthened the idea that UVB irradiation of fibroblasts may result in interaction of CK2 with p70S6k a short time after irradiation. In a preliminary experiment using a co-immunoprecipitation technique we indeed could show a physical interaction between protein kinase CK2 and p70S6k at 20, 30, 40 and 60 min upon UVB irradiation (results not shown). However, in this experiment p70S6k and CK2 also interacted in non-irradiated cells, showing the same intensity of the chemiluminescence signal as for the irradiated fibroblasts (results not shown). For this reason, interaction of p70S6k and CK2 may not be an exclusively UVB-dependent event. A possible explanation is that UVB irradiation does not result in an increase in the CK2 protein amount bound to p70S6k, but rather results in an increase in CK2 activity, at least within the studied time range shortly after the initial irradiation events. However, it cannot be excluded that the interaction of the proteins becomes stronger upon UVB irradiation. However, the different time kinetics of CK2 (this study) and p70S6k [12] suggest that CK2 is an upstream regulator of p70S6k and therefore in conjunction with its effect on p53 activation a component of the DNA-damage-dependent signalling pathway leading to UVB-mediated induction of MMP-1 and MMP-3. Further studies are needed to prove this hypothesis.

Our results may fit to an earlier concept and published data that convincingly show that CK2 is able to form stable complexes with several substrates through interactions that involve either its α subunit or β subunit [26]. As CK2 is located in the cytosol as well as in the nucleus the potential interacting partners of CK2 comprise a large number of nuclear proteins; proteins involved in signalling such as Raf, Mos and phosphatase 2A, and proteins involved in DNA damage, such as p53 and $p21^{WAF1/CIP1}$ [26]. More recently, it was shown that CK2α subunit formed a stable ATP/GTP-dependent complex with a phosphatidic acid-preferring phospholipase 1A by a novel mechanism [53].

Collectively, we have identified the protein kinase CK2 as a novel signalling component activated by low-dose UVB irradiation and which, at least in part, is responsible for the enhanced synthesis of two major MMPs finally contributing to connective-tissue degradation in photoaging and tumour progression. In this study *in vitro* UVB intensities were used that can be absorbed readily by fibroblasts within the skin *in vivo* [54,55]. This, in conjunction with increased UVB fluxes on the skin surface due to stratospheric ozone depletion [2], emphasizes the relevance of our data and may stimulate the development of sunscreen formulations and agents that reliably protect from UVB-irradiation-induced noxious signalling events.

We thank Dr E. M. Chambaz and Dr C. Cochet (INSERM, Grenoble, France) for supplying us with the kinase-inactive mutant of CK2α catalytic subunit (CK2α-K68A-HA), and Dr P. Herrlich (Forschungszentrum Karlsruhe, Institute of Toxicology and Genetics), for the cDNA clone for human MMP-1. This work was supported by grants from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF; Förderkennzeichen 07UVB55B/1), the European Community (ENV4-CT96-172) and the Köln Fortune Program (Faculty of Medicine, University of Köln, Köln, Germany).

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Received 17 January 2002/14 March 2002; accepted 19 April 2002