Activation of p70 Ribosomal Protein S6 Kinase Is an Essential Step in the DNA Damage-dependent Signaling Pathway Responsible for the Ultraviolet B-mediated Increase in Interstitial Collagenase (MMP-1) and Stromelysin-1 (MMP-3) Protein Levels in Human Fibroblasts*

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Ultraviolet B (UVB) irradiation has been shown to stimulate the expression of matrix-degrading metalloproteinases via generation of DNA damage and/or reactive oxygen species. Matrix-degrading metalloproteinases promote UVB-triggered detrimental long term effects like cancer formation and premature skin aging. Here, we were interested in identifying components of the signal transduction pathway that causally link UVB-mediated DNA damage and induction of matrix-degrading metalloproteinase (MMP)-1/interstitial collagenase and MMP-3/stromelysin-1 in human dermal fibroblasts in vitro. The activity of p70 ribosomal S6 kinase, a downstream target of the FK506-binding protein-12/rapamycin-associated protein kinase (FRAP) kinase (RAFT1; mTOR), were identified to be 0.8-fold, and MMP-1 and MMP-3 protein levels 2.4- and 11.5-fold increased upon UVB irradiation compared with mock-irradiated controls. The FRAP kinase inhibitor rapamycin and the DNA repair inhibitor aphidicolin significantly suppressed the UVB-mediated increase in p70 ribosomal S6 kinase activity by 50–65% and MMP-1 and MMP-3 protein levels by 34–68% and 42–88% compared with UVB-irradiated fibroblasts. By contrast, the interleukin-1β-mediated increase in MMP-1 and MMP-3 protein levels could not be suppressed by rapamycin. Collectively, our data suggest that the FRAP-controlled p70 ribosomal S6 kinase is an essential component of a DNA damage-dependent, but not of the interleukin-1/cell membrane receptor-dependent signaling.

In past years, two signaling pathways for the mammalian ultraviolet (UV) response have been identified. The first pathway comprises UV irradiation-dependent generation of reactive oxygen species near or within the cell membrane. UV-generated reactive oxygen species activate receptor tyrosine kinases and protein kinases at the inner surface of the plasma membrane and elicit a signaling cascade, which activates transcription factors and the transcription of defined genes (1–3). The signaling cascade of the second pathway originates in the cell nucleus with indirect (oxidative) (4) or direct DNA damage as the primary signal, followed by passage of secondary “signals” to the cytoplasm, thereby activating specific signaling pathways, which eventually return to the nucleus and induce changes in transcription factors and gene expression (1, 5, 6).

In view of the compelling evidence that the increase in ultraviolet-B (UVB) irradiation (280–320 nm) on earth due to stratospheric ozone depletion represents a major environmental threat to the skin (7–9), we and others focused our studies on signaling cascades which mediate noxious UVB effects. UVB irradiation has recently been shown to generate lipid peroxides and hydroxyl radicals (HO·) (10–13) in skin cells with detrimental long term effects like cancer formation (9, 14) and premature aging (9, 15, 16). The major types of DNA lesions directly generated by UVB and artificial UVC irradiation predominantly occur at dipyrimidine sites with the formation of cyclobutane pyrimidine dimers (CPD) and pyrimidine (6–4)pyrimidone photoproducts (17, 18), which per se may represent initial steps in UV-induced photocarcinogenesis (9).

Mammalian cells react to UVB irradiation with a number of genetic changes including the expression of distinct genes (19, 20) involved in signal transduction pathways, cell cycle control, tumor promotion, and progression. UVB-inducible genes comprise several proteinases, among them members of the matrix-degrading metalloproteinase (MMP) superfamily (9, 16, 21–23). As to their proteolytic activity, various UVB-induced matrix-degrading metalloproteinases in dermal fibroblasts contribute to the breakdown of dermal interstitial collagen and other connective tissue components and, thus, besides initiating cutaneous photoaging (15, 16, 21, 22, 24) promote tumor invasion and metastasis (25, 26).

Over the past years, many studies have focused on the complex regulatory molecular and cellular mechanisms underlying UVB-mediated up-regulation of MMPs. Recently, several components of the intracellular signal transduction pathways including c-Jun and c-Jun N-terminal kinases have been identified to be involved in the UVB-mediated induction of matrix-
degrading metalloproteinases (16, 21, 22). Furthermore, iron-dependent generation of cell membrane-derived lipid peroxides and hydroxyl radicals upon UVB irradiation were found to be involved in the downstream signaling pathway, finally leading to the induction of interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) (21), two major members of the MMP family (16, 21, 27, 28). These and other data (3) provide evidence that UVB-initiated signaling events at the cell membrane may feed into the classical protein kinase cascades; however, the responsible chromophore(s) within or at the membrane has (have) not yet been identified. In addition, there is independent evidence that induction of cytokines and of delayed UV-responsive genes like interstitial collagenase (MMP-1) require DNA as a primary chromophore for the absorption of UVB or UVC irradiation with subsequent formation of DNA damage. In fact, cyclobutane pyrimidine dimers have been shown to play an important role in these processes (29–31).

Only limited information is available regarding (i) the components of DNA damage-dependent signal cascade(s) occurring upstream of the activation of specific target genes, and (ii) their potential involvement in membrane-dependent signal transduction pathways. Understanding the signaling events that are initiated by UVB-mediated DNA damage and that result in the up-regulation of matrix-degrading metalloproteinases is of considerable interest, since it may provide ultimate clues for mechanisms underlying connective tissue degradation in pathological states. Recently, the DNA protein kinase FRAP (FKBP6-binding protein-12 (FKBP)/rapamycin-associated protein kinase) (32–34) also belonging to the phosphoinositide 3-kinase-like family (35, 36) such as the DNA damage-dependent protein kinase (36, 37) was identified to play a central role in the signaling pathway which activates transcription of tumor necrosis factor α (TNFα) following UVB-induced DNA damage (38, 39). Inspired by these results, we have set out to identify components of the signal transduction pathway that causally link UVB-mediated DNA damage to the induction of matrix-degrading metalloproteinases (MMP). Using the FRAP inhibitor rapamycin (40) and/or the specific nucleotide and long patch base excision repair inhibitor aphidicolin (41, 42) prior to low UVB irradiation of human dermal fibroblasts, we found that the activity of p70 ribosomal protein S6 kinase (p70S6k), the downstream target of FRAP kinase, as well as MMP-1 and MMP-3 protein levels were significantly reduced. These data may indicate that p70S6k and FRAP are essential components in the UVB-induced signal transduction pathway at least in part responsible for the induction of distinct MMPs. By contrast, interleukin-1-initiated/cell membrane receptor-dependent induction of MMP-1 and MMP-3 was not affected by rapamycin and/or aphidicolin further supporting the view that p70S6k and its upstream regulator FRAP are essentially required for the DNA damage-dependent, but not for the interleukin-1-initiated signaling pathway.

EXPERIMENTAL PROCEDURES

Reagents—Cell culture medium and Trizol® reagent were purchased from Life-Technologies, Inc. (Eggenstein, Germany) and fetal calf serum from Sigma (Deisenhofen, Germany) unless otherwise indicated. Human interleukin-1β (IL-1β), specific rabbit polyclonal p70S6k antibody, and components of the DNA assay were from BIOKOL (Hamburg, Germany). MMP-1, MMP-3, and TIMP-1 cDNA/enzyme-linked immunosassay (ELISA) kits (Biotrak) were obtained from Amer- sham Pharmacia Biotech (Braunschweig, Germany) and PhosphoPlus RNA Extraction and Northern Blot Analysis—Total RNA was isolated and subjected to Northern blot analysis and sequencially hybridized with specific cDNA probes for interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), and TIMP-1 as published elsewhere (47, 53). Briefly, after preparation of total RNA from fibroblasts using the Trizol® method, equal amounts (5 μg/lane) were fractionated by size in the dark. The size of the ribonucleic acid (RNA) molecules was calculated from the size and position of the length dispersion curve was calculated as percentage of formazan formation in cells treated with the agents compared with mock-treated cells.

ELISA—MMP-1, MMP-3, and TIMP-1 “sandwich” ELISA assays were performed according to the manufacturer’s protocols (Amersham Pharmacia Biotech) 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 peroxidase substrate. Optical densities were read at 450 nm using a microtiter plate reader L9400 (Sanofi Diagnostics Pasteur, Freiburg, Germany). Concentrations of MMP-1, MMP-3, and TIMP-1 in the samples were determined against standard curves using Graph-Pad Software (San Diego, California).
nitrocellulose filters and vacuum baking (Schleicher & Schuell, Dassel, Germany), hybridizations were performed using denatured α-32P-labeled cDNA probes. Densitometric analysis was performed using the ScanPackII system (Biometra, Gottingen, Germany).

**Immunoprecipitation and S6 Kinase Assay**—The activity of p70 S6 kinase was determined by incorporation of 32P into S6 peptide (AKRRRLSSLRA) as described previously (54) with minor modifications. Briefly, after a 24-h starvation period (0.2% FCS in DMEM), confluent fibroblasts were either serum-stimulated (20% FCS) or irradiated at a dose of 10 mJ of UVB/cm². At different time points thereafter, cells were lysed in ice-cold modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of each aprotinin, leupeptin, and pepstatin, 1 mM activated sodium orthovanadate (Na₃VO₄)). Cell lysates were preclreated (100 μl of protein A-agarose bead slurry/ml of lystate) and thereafter 500 μl of that lysate (1 μg of total protein/μl) were incubated with a specific rabbit polyclonal antibody raised against the C terminus (amino acids 511–526, CFP-MISKPRHELRL) of p70 ribosomal S6 kinase at 4 °C for 2 h. Aliquots of 25 μl/sample were subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and stained with Ponceau S solution to verify equal amounts of total protein in different samples. The immune complex was absorbed to protein A-agarose (50 μl of bead slurry) for 1 h at 4 °C and washed twice with ice-cold phosphate-buffered saline and once with assay dilution buffer (ADB) (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EDTA, 1 mM Na3VO₄, 1 mM dithiothreitol). Half the volume of the resulting precipitate was suspended in a 50-μl reaction volume composed of 10 μl each of 250 mM substrate peptide in ADB, inhibitor mixture (20 μM protein kinase C, 2 μM protein kinase A, and 20 μM calmodulin kinase inhibitor peptide in ADB), and a magnesium/ATP mixture (10 μCi of [γ-32P]ATP (~300 Ci/mmol) in 75 mM magnesium chloride, 500 μM ATP), and incubated for 15 min at 30 °C with gentle agitation. After centrifugation, 25-μl aliquots per sample supernatants were spotted on Whatman P-81 phosphocellulose paper, washed five times for 5 min with 0.75% phosphoric acid, and finally for 2 min with 96% ethanol. Radioactivity bound to phosphocellulose paper was determined as picomoles of phosphate incorporated into p70 S6 kinase per milligram of total protein.

**Immunoblot Analysis**—Immunoblot analysis was performed according to standard protocols published elsewhere (55) with minor modifications. Briefly, cells were subjected to specific treatment and cell lysates were prepared as described above. Equal amounts of proteins (50 μg/lane) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. The filters were incubated with a 1:1000 dilution of anti-rabbit secondary antibodies conjugated to horseradish peroxidase. Antigen/antibody complexes were visualized by enhanced chemiluminescence (Phototope detection kit) and exposed to Kodak X-Omat AR film.

**RESULTS**

**Cytotoxicity of the FRAP Kinase Inhibitor Rapamycin and the Nucleotide Excision Repair Inhibitor Aphidicolin**—We intended to avoid interference of cytotoxicity from chemical agents. Therefore, MTT assays were performed to determine optimal non-toxic concentrations at which ~80% of the mitotic fibroblasts survived at least 24 h after incubation with rapamycin or aphidicolin with no change in morphology. The maximal concentrations, which fit the above mentioned requirement, were 500 nM for rapamycin and 10 μM for aphidicolin (Fig. 1A). For subsequent experiments using the chemical agents alone or in combination, lower concentrations of 100 nM rapamycin and 5 μM aphidicolin were applied since combinations of the agents at these concentrations plus UVB irradiation maintained viability between 90% and 100% (Fig. 1B). Combinations of higher concentrations of the chemical compounds plus UVB irradiation reduced viability below 80% (data not shown).

**UVB Dose-dependent Increase in CPD Correlates with an Increase in MMP-1 and MMP-3 mRNA Levels**—Confluent human dermal fibroblasts pretreated with the repair inhibitor aphidicolin were irradiated at low doses of 10 and 20 mJ/cm² (47). Thereafter, the initial CPD frequency was measured by high resolution pulsed-field gel electrophoresis and an identically treated set of cells was subjected to Northern blot analysis for determination of specific MMP-1 mRNA levels 24 h after irradiation (Fig. 2). Following UVB irradiation, an 8.2-fold increase at a dose of 10 mJ/cm² and a 12.6-fold increase in CPD frequency at a dose of 20 mJ/cm² was found compared with mock-irradiated fibroblasts. Twenty-four h after irradiation at UVB doses of 10 and 20 mJ/cm², a 5.1- and 6.9-fold increase in MMP-1 mRNA levels, and a 2.7- and 4.6-fold increase in MMP-3 mRNA levels (47) was observed (Fig. 2). These data provide circumstantial evidence for a correlation between UVB-mediated DNA damage and the induction of MMP.

**UVB-mediated Increase in Interstitial Collagenase (MMP-1) and Stromelysin-1 (MMP-3) Protein Levels Can Partly Be Suppressed by Rapamycin and Aphidicolin**—In order to study which components of the UVB-dependent and DNA damage-initiated signaling pathway are causally involved in the induction of matrix-degrading metalloproteinases, we here used the DNA repair inhibitor aphidicolin (41) and rapamycin, an immunosuppressant which (via the FKBP) specifically binds and inhibits the FRAP kinase (32, 40), a recently identified DNA protein kinase (36, 38). Confluent fibroblast monolayer cultures were incubated with non-toxic concentrations of rapamycin and/or aphidicolin prior to UVB irradiation at a dose of 10 mJ/cm². Twenty-four h after irradiation cell culture supernatants were subjected to ELISA analysis (Fig. 3). UVB irradiation resulted in a 2.4- and 11.5-fold increase in MMP-1 (Fig. 3A) and MMP-3 (Fig. 3B) protein levels, respectively, compared with mock-irradiated controls. Preincubation of fibroblasts with rapamycin and/or aphidicolin prior to UVB irradiation reduced MMP-1 protein levels by 34–68% (Fig. 3A) and MMP-3 protein levels by 32-67% (Fig. 3B). These data provide circumstantial evidence for a correlation between UVB-mediated DNA damage and the induction of MMP.
levels by 42–88% (Fig. 3B) compared with the UVB-irradiated controls. Similar results were obtained under identical experimental conditions after UVB irradiation at a dose of 20 mJ/cm² (data not shown). Interestingly, the inhibitory effect of aphidicolin on UVB-induced MMP-1 and MMP-3 protein levels was much more pronounced compared with rapamycin, and could not be further enhanced by combined treatment with rapamycin. By contrast, TIMP-1 protein levels were unchanged under these experimental conditions (Fig. 3C).

The Interleukin-1β-mediated Increase in Intermembrane Collagenase (MMP-1) and Stromelysin-1 (MMP-3) Protein Levels Is Independent of Rapamycin—To address the question of whether rapamycin affects the IL-1β-dependent, cell membrane-initiated induction of MMP-1, MMP-3, and their tissue inhibitor TIMP-1, confluent fibroblasts were preincubated with 100 nM rapamycin prior to IL-1β treatment (Fig. 4). As described previously, IL-1β concentrations of 0.1 and 1.0 ng/ml, respectively, were used to induce MMP-1, MMP-3, and TIMP-1 (56, 57). Following IL-1β treatment at a concentration of 0.1 ng/ml, an 1.6-fold increase in MMP-1 protein levels was measured compared with the mock-treated controls, while a concentration of 1.0 ng/ml resulted in a 2.3-fold increased MMP-1 protein levels (Fig. 4A). In parallel experiments, MMP-3 protein levels were similarly elevated 2.8- and 3.3-fold (Fig. 4B). In contrast to the rapamycin effect on UVB-irradiated cells (Fig. 3), preincubation of fibroblast monolayer cultures with rapamycin prior to IL-1β treatment did not result in a significant reduction of MMP-1 and MMP-3 protein levels (Fig. 4, A and B). In addition, IL-1β treatment had no effect on TIMP-1 protein levels (Fig. 4C).

Aphidicolin but Not Rapamycin Suppresses UVB-mediated Increase in Intermembrane Collagenase (MMP-1) mRNA Levels—In order to study whether rapamycin and/or aphidicolin exert their effects on the level of transcription, confluent fibroblast monolayer cultures were preincubated with the above-mentioned agents prior to UVB irradiation as described above. 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 (Fig. 5A). UVB irradiation at a dose of 10 mJ/cm² resulted in a 4.5-fold increase in MMP-1 steady-state mRNA levels compared with mock-irradiated fibroblasts, while TIMP-1 mRNA levels remained unchanged. Interestingly, rapamycin did not reveal any significant effect on the UVB-induced MMP-1 and TIMP-1 mRNA levels. By contrast, the DNA repair inhibitor aphidicolin alone or in combination with rapamycin significantly reduced MMP-1 mRNA levels by 66% as compared with UVB-irradiated controls (Fig. 5A). Again, TIMP-1 mRNA levels remained unchanged under these experimental conditions.

To elucidate the effect of rapamycin on receptor/cell membrane-dependent induction of specific mRNA levels of MMP-1 and TIMP-1, fibroblasts were preincubated with rapamycin prior to treatment with exogenously added IL-1β, total RNA was isolated and subsequently subjected to Northern blot analysis to determine specific MMP-1 and TIMP-1 mRNA levels (Fig. 5B). Low constitutive levels of MMP-1 and TIMP-1 mRNA were detected. IL-1β treatment at concentrations of 0.1 and 1.0 ng/ml led to a 3.3- and 4.5-fold increase in MMP-1 mRNA levels compared with mock-treated controls. Similar to the unremarkable effects of rapamycin on IL-1β-dependent MMP-1 protein levels, rapamycin did not affect increased MMP-1 steady-state mRNA levels after IL-1β treatment (Fig. 5A). TIMP-1 mRNA levels also remained unchanged compared with mock-treated controls under identical experimental conditions (Fig. 5B).
Kinase (p70S6k) Activity by Rapamycin and Aphidicolin—The p70S6k is a serine/threonine kinase which phosphorylates 40 S ribosomal protein S6 in response to a number of extracellular stimuli (34, 58–60). FRAP kinase has been discussed to be a direct upstream mediator of p70S6k in different experimental settings (32, 40). Accordingly, rapamycin specifically inhibits FRAP activity (40) and, hence, indirectly also that of p70S6k. To study the involvement of the FRAP-p70S6k pathway in the UVB-mediated increase in MMP-1 and MMP-3 protein levels, confluent serum-starved fibroblasts were UVB-irradiated and subjected to S6 kinase assay at different time points thereafter (Fig. 6A).

Following pretreatment with rapamycin and aphidicolin and subsequent UVB irradiation at a dose of 10 mJ/cm², fibroblasts were subjected to S6 kinase assays at 1 h after irradiation (Fig. 6B). Again, serum-stimulated or UVB-irradiated fibroblasts showed a similarly induced p70S6k activity with a 4.8 ± 0.2-fold increase after serum stimulation and 4.3 ± 0.9-fold increase in p70S6k activity after UVB irradiation. Interestingly, rapamycin and aphidicolin alone or in combination reduced p70S6k activity by 50–65% compared with UVB-irradiated fibroblasts (Fig. 6B).

A low constitutive activity of p70 S6 kinase was detected in mock-irradiated control cells, while serum-stimulated (20% FCS) fibroblasts, used as positive control (58), showed a 4.5 ± 0.1-fold increase in p70S6k activity at 30 min after serum incubation compared with mock-treated controls (Fig. 6A). Following UVB irradiation, a time-dependent increase in p70S6k activity was observed, which peaked at 1 h after irradiation with a 4.3 ± 0.8-fold increase compared with mock-treated controls. This activity gradually decreased 24 h after irradiation without reaching basal levels of mock-treated controls (Fig. 6A).

Following pretreatment with rapamycin and aphidicolin and subsequent UVB irradiation at a dose of 10 mJ/cm², fibroblasts were subjected to S6 kinase assays at 1 h after irradiation (Fig.
fibroblasts (Fig. 6B). The inset (Fig. 6B) shows a representative protein stain with Ponceau S, which verifies almost identical amounts of total protein for all loaded samples.

By contrast, independent of preincubation with rapamycin, IL-1β treatment of fibroblasts did not result in any significant change in p70S6k activity over mock-treated control cells at 1 h after IL-1β treatment (p < 0.41) (Fig. 6C). This finding is in contrast to previously published data showing that stimulation of T cells with cytokines like IL-1 and IL-2 resulted in a maximal induction of p70S6k protein synthesis and activity at 1 h after treatment (61, 62).

UVB-mediated Activity of p70 S6 Ribosomal Kinase (p70S6k) Is Independent of Phosphorylation at Distinct Phosphorylation Sites—The activity of p70S6k is controlled by multiple phosphorylation events located within the catalytic and autoinhibitory (pseudosubstrate) domain of this enzyme. All phosphorylation sites display the motif Ser/Thr-Pro (63, 64).

In order to study UVB-dependent phosphorylation at distinct phosphorylation sites in the pseudosubstrate region of the active kinase, confluent serum-starved fibroblasts were UVB-irradiated and subjected to immunoblot analysis at different time points after irradiation (Fig. 7). Two phosphospecific antibodies exclusively recognizing phosphorylated p70S6k at either Ser-411 or Thr-421/Ser-424 and a phosphorylation state-independent p70S6k antibody were used. Thirty min after serum stimulation of fibroblasts (20% FCS) (58) phosphorylation at Ser-411 and Thr-421/Ser-424 (C (197)) was detected. By contrast, mock-irradiated (C(B)) and UVB-irradiated cells did not show any phosphorylation at these sites at 0.5, 1, 2, 4, and 24 h after irradiation, while unphosphorylated p70S6k was clearly detectable. Furthermore, experiments using the phosphorylation-state independent p70S6k antibody did not indicate any significant differences in p70S6k protein levels in either mock-irradiated, FCS-stimulated, or UVB-irradiated fibroblasts, suggesting that UVB irradiation has no effect on the overall protein synthesis of p70S6k. Identical amounts of total protein have been transferred to nitrocellulose filters as deduced from identical intensities of the nonspecific band (Fig. 7) and Ponceau staining (data not shown).
In this study we have focused on the identification of components of the DNA damage-dependent signal transduction pathway being causally involved in the induction of two major members of the MMP family following UVB irradiation. Apart from independent evidence that interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) play a major role in multistage processes of carcinogenesis and cutaneous photoaging (16, 21, 24, 65–67) and that UVB-mediated DNA damage is an essential prerequisite for the development of skin cancer (67, 68) and premature aging (67, 69), pyrimidine dimers in transcriptionally active DNA have previously been shown to transduce signals, finally resulting in transactivation of UV-responsive genes like interstitial collagenase (29, 30). However, little is known about DNA damage-dependent signaling components being activated upstream from these genes of interest. Herein, we were able to show that the activity of p70 ribosomal S6 kinase (p70S6k), but not p70S6k protein synthesis, is UVB-dependently stimulated and that p70S6k is essentially required for the UVB/DNA damage-initiated signal cascade(s) leading to the induction of MMP-1 and MMP-3.

For the mammalian UV response, two signaling pathways have been described that either originate at the plasma membrane being initiated by reactive oxygen species and/or cytokine receptor clustering (1, 21, 31, 70) or originate in the nucleus by indirect (oxidative) (4) or direct DNA damage (1, 5, 6, 29, 30) as the primary signal. We earlier found that ferrous/ferric iron-driven generation of lipid peroxides and hydroxyl radicals with subsequent activation of c-Jun N-terminal kinase 2 and c-Jun may play a role in the UVB-induced cell membrane-dependent signaling pathway, eventually leading to increased interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) mRNA levels in cultured human dermal fibroblasts (21). We herein report that the dose-dependent increase in cyclobutane pyrimidine dimers correlates with dose-dependent increase in MMP-1 and MMP-3 mRNA levels after UVB irradiation of fibroblasts at low doses of 10 and 20 mJ/cm². These findings further strengthen the idea that several molecular origins of UVB signaling pathways (as has earlier been postulated) may coexist (2, 3, 5, 30, 71). Besides further characterization of these molecular origins of UVB signaling, we and others are interested in the identification of molecular components downstream of these initiating events and upstream of the genes of interest. In this report, we particularly pursued the question of whether signal transduction pathways either initiated by DNA damage or membrane-dependent events make use of the same molecular components finally leading to the induction of two prominent members of the matrix-degrading metalloproteinase family. Inspired by recent findings (38), we have used the FRAP inhibitor rapamycin (38, 40, 72, 73) to identify a possible role of FRAP and its downstream target p70S6k in the UVB/DNA damage-dependent up-regulation of matrix-degrading metalloproteinases.

A major finding of this study is that non-toxic concentrations of rapamycin, in fact, significantly reduced interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) protein levels following low dose UVB irradiation suggesting that FRAP kinase may represent the missing molecular link between UVB-initiated DNA damage and enhanced MMP-1 and MMP-3 protein levels. By contrast, protein levels of the major tissue inhibitor of MMP-1 and MMP-3, TIMP-1, remained unaffected by rapamycin providing evidence that UVB-initiated DNA damage does not regulate TIMP-1 and, thus, promote proteolysis and subsequent connective tissue degradation following UVB irradiation (16).

As rapamycin selectively reduced specific protein levels, but not mRNA levels of MMP-1 and MMP-3 upon UVB exposure, we were specifically interested to determine the role of the p70S6k, a rapamycin-sensitive downstream target of FRAP with translation regulating properties for a subset of mRNAs (72, 74–77) in the herein reported novel UVB/DNA damage-initiated signaling pathway. Our major finding is that low dose UVB irradiation resulted in a significant increase in p70S6k activity, which, preceding the down-regulation of specific MMP-1 and MMP-3 protein levels, was almost completely abrogated by rapamycin. These data suggest that p70S6k, in fact, is a key regulator in the novel UVB/DNA damage-dependent signaling pathway. Thus, in addition to its well known activation by growth factors, hormones, serum and phorbol esters (32, 34, 60, 72, 75, 77), we here report a novel facet of p70S6k activation, namely by UVB-induced DNA damage, and furthermore, regarding its regulatory role in the translation of matrix-degrading metalloproteinases describe a novel effector function of p70S6k which eventually contribute to connective tissue degradation. In fact, enhanced proteolysis of interstitial collagenas (the preferential substrate for MMP-1) has recently been reported in UVB-irradiated human skin in vivo (78, 79).

In contrast to UVB irradiation, IL-1β treatment neither resulted in a significant increase in p70S6k activity nor did rapamycin show any significant effect further supporting the specificity of the UVB/DNA damage-initiated signaling pathway and suggesting that cell membrane/receptor- and DNA damage-initiated pathways are distinct from each other or a general pathway bifurcates upstream from rapamycin-sensitive signal transduction components.

The activation of p70S6k is due to a complex series of phosphorylation events, which are thought to be directly mediated by upstream regulators such as mTOR/FRAP (32, 64, 72) and/or still unidentified effector kinases(s) (64). As phosphorylation sites displaying the Ser/Thr-Pro motif within the catalytic and autoinhibitory (pseudosubstrate) domain of p70S6k including Ser-411, Thr-421, and Ser-424 have been reported to be responsible for the activation of p70S6k (63, 64, 75, 80), we addressed the question of whether the above mentioned sites became phosphorylated upon UVB irradiation. Prior to studying UVB effects, we have determined p70S6k phosphorylation in serum-stimulated fibroblasts, which served as a positive control. Quiescent fibroblasts became specifically phosphorylated at the above mentioned sites in response to serum with concomitant increase in p70S6k activity, supporting earlier published results (64, 80). By contrast, although we observed an overall increase in p70S6k activity, we could not detect any UVB-dependent phosphorylation of Ser-411, Thr-421, and Ser-424, suggesting that other phosphorylation sites in the p70S6k protein may play a role in the UVB/DNA damage-dependent activation of p70S6k. Thus, our results fit to a recently published concept, which (based on deletion constructs of the carboxyl terminus including the complete autoinhibitory region of p70S6k) convincingly shows that, independent of this region, p70S6k became phosphorylated upon UVB/ DNA damage-dependent up-regulation of matrix-degrading metalloproteinases.

A number of years it has been well known that DNA-damaging agents such as ultraviolet radiation (UVR) activate transcription factors such as Fos and Jun, which, heterodimerized, constitute the AP-1 transcription factor complex and subsequently induce genes like MMP-1 and MMP-3 (5, 19, 20, 21, 27–30). Previously, repair processes have been demonstrated to be connected with gene induction. Hence, repair is highly selective for transcribed DNA strands in active genes and directly coupled to the transcription apparatus (83–85). In addition,
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UV-generated photoproducts in transcribed DNA regions per se may induce gene transcription, suggesting that neither ongoing repair nor specific DNA repair intermediates are required for transcriptional activation of delayed genes such as MMP-1 (30). In this study, we were interested in the identification of components of DNA damage/repair-dependent pathway leading to induction of matrix-degrading metalloproteinases (MMP). Therefore, we have used the chemical compound aphidicolin known for its specific nucleotide and long patch base excision repair inhibiting properties (41, 42) to modulate UVB-induced MMP-1/-3 expression. Following pretreatment of dermal fibroblasts with a non-toxic aphidicolin concentration prior to UVB irradiation, we found that UVB-mediated increase in MMP-1 mRNA level was almost completely abrogated. In addition, aphidicolin suppressed the UVB-induced specific protein levels of MMP-1 and MMP-3 suggesting that both UVB-enhanced transcription and translation of MMP-1 and MMP-3, respectively, may at least in part depend on excision repair events which occur subsequent to UVB-mediated DNA damage. A possible clue why the DNA repair inhibitor aphidicolin results in almost complete suppression of the UVB-induced MMP-1 and MMP-3 transcription, may come from the dual role of certain proteins in repair and transcription (86). The importance of this dual function in the regulation of distinct genes, such as ICAM-1, has recently been reported (87). Whether a similar mechanism may be active in aphidicolin suppression of the UVB-induced transcription of MMP-1 and MMP-3 genes remains to be resolved. Regardless of the exact mechanism, our finding that increase in UVB-mediated p70S6k activity, similar to MMP-1 and MMP-3 protein levels, was almost completely abrogated upon pretreatment fibroblasts with aphidicolin prior to irradiation supports the idea that UVB-mediated p70S6k activation depends on DNA repair-dependent mechanism(s).

Collectively, we have identified the FRAP/p70S6k signaling cascade as a novel pathway regulated by UVB-induced DNA damage/repair, which is at least in part responsible for the enhanced synthesis of two major matrix-degrading metalloproteinases finally contributing to connective tissue degradation in photoaging and tumor progression. UVB intensities as used in this study in vitro can be absorbed readily by fibroblasts within the skin in vivo (88, 89). This, in conjunction with increased UVB fluxes on the earth surface due to stratospheric ozone depletion (7) points to the relevance of our data and may stimulate the development of sunscreen formulations that reliably protect from DNA damage/repair-induced noxious signaling events.

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