Nitric oxide (NO) represents a short lived mediator that pivotally drives keratinocyte movements during cutaneous wound healing. In this study, we have identified p68 DEAD box RNA helicase (p68) from an NO-induced differential keratinocyte cDNA library. Subsequently, we have analyzed regulation of p68 by wound-associated mediators in human and murine keratinocytes. NO, serum, growth factors, and pro-inflammatory cytokines were potent inducers of p68 expression in the cells. p68 was constitutively expressed in the epithelial compartment of murine skin. Upon injury, we found a transient down-regulation of overall p68 protein in wound tissue. However, p68 did not completely disappear during early wound repair, as we found an expression of p68 protein in isolated wound margin tissue 24 h after wounding. Moreover, immunohistochemistry and cell fractionation analysis revealed a restricted localization of p68 in keratinocyte nuclei of the developing epithelium. Accordingly, cultured keratinocytes also showed a nuclear localization of the helicase. Moreover, confocal microscopy revealed a strong localization of p68 protein within the nucleoli of the cells. Functional analyses demonstrated that p68 strongly participated in keratinocyte proliferation and gene expression. Keratinocytes that constitutively overexpressed p68 protein were characterized by a marked increase in serum-induced proliferation and vascular endothelial growth factor expression, whereas down-regulation of endogenous p68 using small interfering RNA markedly attenuated proliferation and vascular endothelial growth factor expression. Altogether, our results suggest a tightly controlled expression and nucleolar localization of p68 in keratinocytes in vitro and during skin repair in vivo that functionally contributes to keratinocyte proliferation and gene expression.

The story of RNA helicases started in 1980, as a yet unknown 68-kDa-sized protein was identified by its strong cross-reactivity with an antibody that has been raised against the simian virus 40 large T antigen (1). A few years later, the cDNA was cloned, and subsequent sequence determination revealed a 68-kDa protein that was characterized by a marked homology to the eucaryotic initiation factor-4A (2). The strikingly conserved motifs of both eucaryotic initiation factor-4A and the identified 68-kDa protein led to the proposal of a novel “DEAD” box family of proteins (3). To date, it is now well established that the increasing number of DEAD box proteins are structurally characterized by eight conserved motifs from which the Asp-Glu-Ala-Asp (DEAD) motif has been used to determine the name of the protein family (3). Subsequent biochemical studies revealed that members of the DEAD box family of proteins were characterized by their potency to unwind double-stranded RNA molecules in an energy-dependent fashion through the hydrolysis of NTP and thus act as prototypical RNA helicases in vitro (4). In particular, eucaryotic initiation factor-4A and the p68 DEAD box RNA helicase (p68) have been shown to catalyze the separation of short RNA duplexes in an ATP-dependent fashion in vitro (5, 6). However, as extended and continuous double-stranded RNA molecules are rare and not of functional significance in biological systems, it is now assumed that DEAD box proteins, including p68, are involved in disintegration or rearrangement of RNA-protein interactions rather than in a processive RNA unwinding activity (7–9). Accordingly, it is now well established that DEAD box proteins participate in important biological processes that involve RNA interactions such as transcription, ribosome biogenesis, pre-mRNA splicing and editing, RNA export to the cytoplasm, translation, and RNA degradation (4). Moreover, nearly all RNA molecules tend to fall into kinetic traps, as they fold into their lowest energy structure by forming intramolecular double helices (10). Thus, the physiologic functions of RNA helicases might even be important in more general terms, as they might serve as RNA chaperones that facilitate the formation of correct RNA structures by prevention or reversal of misfolded states (11). Regarding the important functions that are assigned to RNA helicases, it is not surprising to recognize a strong conservation of p68 protein during evolution. Human and murine p68 proteins share a 98% identity (12), and even the Saccharomyces cerevisiae p68 homologue Dpb2p exhibits a 55% identity to the human protein. Moreover, protein function appeared to be even more conserved, as S. cerevisiae Dpb2 null cells can be rescued by the human p68 protein (13, 14). Nevertheless, information that connects the knowledge of p68 in vitro functions to physiological or pathophysiological conditions in vivo is rather unsatisfactory. Serum inducibility of p68 in Swiss 3T3 fibroblasts indicated a functional clue to cellular proliferation (15). However, expression levels of p68 protein did not always correlate to the proliferation status in adult tissue, although there is evidence that p68 expression is connected to organ differentiation and maturation of the fetus (15). Additionally, p68 has been demonstrated to be overexpressed in nuclei of epithelial...
cells under hyperproliferative conditions of colorectal adenocarcinoma (16). In this study, we have identified p68 as a novel NO-regulated gene in skin keratinocytes. Additionally, we found keratinocyte p68 expression to be induced by growth factors and cytokines that control keratinocyte movements in the context of cutaneous wound healing. Within the repair process, a controlled but strong proliferation of wound margin keratinocytes drives the re-epithelialization of the wounded area. Here we demonstrate that p68 is regulated during skin repair and that p68 is functionally connected to keratinocyte proliferation and vascular endothelial growth factor (VEGF) expression in the cells.

EXPERIMENTAL PROCEDURES

Animals—Female C57BLKS mice were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany). Female C57BLKS-iNOS−/− mice were the generous gift of G. Geisslinger, Frankfurt, Germany. Mice were maintained under a 12-h light/12-h dark cycle at 22 °C until they were 8 weeks of age. At this time they were caged individually, monitored for body weight, and weighed as described below.

Wounding of Mice—Wounding of mice was performed as described previously (17, 18). Briefly, mice were anesthetized with a single intraperitoneal injection of ketamine (80 mg/kg body weight)/xylazine (10 mg/kg body weight). The hair on the back of each mouse was cut, and the back was subsequently wiped with 70% ethanol. Six full-thickness wounds (5 mm in diameter, 3–4 mm apart) were made on the back of each mouse by excising the skin and the underlying panniculus carnosus. The wounds were allowed to form a scar. Skin biopsy specimens were obtained from the animals 5, 10, and 16 h, or 1, 3, 5, 7, and 13 days after injury. At each time point, an area which included the scar, the complete epithelial and dermal compartments of the wound margins, the granulation tissue, and parts of the adjacent muscle and subcutaneous fat tissue was excised from each individual wound. As a control, a similar amount of skin was taken from the backs of nonwounded mice. Frozen sections were used to perform immunohistochemistry, RT-PCR-Select cDNA subtraction, and RT-PCR. PCR-Select cDNA subtraction kit (Clontech) was used for cDNA subtraction, and RT-PCR were performed according to the manufacturer’s instructions. Combinant bacterial clones were identified by isopropyl-1-thio-β-D-galactopyranoside/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) co-incubation and subsequently grown overnight in 384-well plates. Equal amounts of the overnight bacterial suspension were spotted onto nylon membranes in duplicate and grown overnight in 384-well plates. 

RNA Isolation and RNase Protection Analysis—RNA isolation and RNase protection assays were carried out as described previously (18, 19). Briefly, 20 μg of total RNA from wounded or nonwounded skin was used for RNase protection assays. RNA probes were cloned into the transcription vector pBluescript II KS (+) (Stratagene, Heidelberg, Germany) and linearized. An antisense transcript was synthesized in vitro by using T3 or T7 RNA polymerase and [α-32P]UTP (800 Ci/mmol). RNA samples were hybridized at 42 °C overnight with 100,000 cpm of the labeled antisense transcript. Hybrids were digested with RNases A and T1 for 1 h at 30 °C. Under these conditions, every single mismatch was recognized by the RNases. Protected fragments were separated on 5% acrylamide, 8 μm urea gels and analyzed by using a PhosphorImager (Fuji, Straubenhardt, Germany). RNases A and T1 were from Roche Applied Science. The murine cDNA probes were cloned using RT-PCR. The probes corresponded to nt 551–785 (for murine p68, GenBank™ accession number NM_007840) or nt 163–317 (for glyceraldehyde-3-phosphate dehydrogenase, GenBank™ accession number NM_000466) of the published sequences. For detection of the human p68 transcript, the identified sequence was used (nt 1891–2161 of the published sequence, GenBank™ accession number NM_004396).

Preparation of Protein Lysates and Cell Fractionation—Skin and cell culture samples were homogenized in lysis buffer (1% Triton X-100, 20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 15 μg/ml leupeptin). The extracts were cleared by centrifugation (17, 20). To obtain nuclear and cytoplasmic fractions of cultured cells and wound tissues, samples were homogenized in lysis buffer (1×, without Triton). Cellular fractions were generated by a single centrifugation step at 100,000 × g for 60 min with an Optima TLX Ultracentrifuge (Beckman Instruments, Munich, Germany). Fractions were subsequently analyzed using the Roth Nanoquant Protein Assay (Bradford method).

Immunoblot Analysis— Fifty to hundred μg of total protein from skin, cellular lysates, or cell fractions were separated by using SDS-gel electrophoresis. After transfer to a polyvinylidene difluoride membrane, p68 protein was subsequently detected by using a polyclonal antibody generated against human p68. The anti-p68 antibody has been generated in rabbits using a peptide sequence (LYSNPQNYQVPRODGRCY-COOH) that allows immunodetection of both human and murine p68 protein. The monoclonal antibody against murine nucleolin and the polyclonal antibody against human proliferating cell nuclear antigen were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The anti-GFP antibody was from Roche Applied Science. A secondary antibody coupled to horseradish peroxidase and the ECL detection system (Amersham Biosciences, Buckinghamshire, U.K.) were purchased from Santa Cruz Biotechnology. An antiserum against human p68 was used for immunodetection.

Cell Culture—Quiescent human HaCaT (21) and murine PAM 212 epidermal keratinocytes were stimulated with GSNO (250 and 500 μM), spermine-NONOate (SpNONOate, 1 M), and 12-O-tetradecanoylphorbol-13-acetate (TPA, 1 M). HaCaT and PAM 212 keratinocytes were maintained under a 12-h light/12-h dark cycle at 37 °C in DMEM containing 10% fetal calf serum. For stimulation with GSNO, the medium was replaced by fresh medium containing 10 mM GSNO, and the cells were incubated for 1 h. At each time point, samples were harvested and processed as described under “Cell Culture.” Murine keratinocytes were stimulated with 100 ng/ml TPA. 

Generation of p68 Overexpressing Keratinocyte Cell Lines—The human p68 RNA helicase cDNA (GenBank™ accession number NM_004396) was amplified from HaCaT keratinocytes using 5′-CCA TGT CGG GTT ATT CCA GTG ACC-3′ as a 5′-primer and 5′-GGC TTA TTG GGA ATA TCC-3′ as a 3′-primer and cloned into the EcoRV site of the pBluescript II KS (+) vector (Stratagene, Heidelberg, Germany). To verify the correct sequence, both strands were sequenced.

ENZYMATIC SEQUENCING:

1. The abbreviations used are: VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; FCS, fetal calf serum; GFP, green fluorescent protein; GSNO, S-nitrosoglutathione; HEK, human embryonal kidney cells; IFN, interferon; IL, interleukin; KGF, keratinocyte growth factor; p68, p68 DEAD box RNA helicase; siRNA, small interfering RNA; SpNONOate, spermine-NONOate; TGF, transforming growth factor; TNF, tumor necrosis factor; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbert assay; iNOS, inducible nitric-oxide synthase; nt, nucleotides; PBS, phosphate-buffered saline; SOD, superoxide dismutase; CREB, cAMP-response element-binding protein; ORF, open reading frame.

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Generation of p68 Overexpressing Keratinocyte Cell Lines—The human p68 RNA helicase cDNA (GenBank™ accession number NM_004396) was amplified from HaCaT keratinocytes using 5′-CCA TGT CGG GTT ATT CCA GTG ACC-3′ as a 5′-primer and 5′-GGC TTA TTG GGA ATA TCC-3′ as a 3′-primer and cloned into the EcoRV site of the pBluescript II KS (+) vector (Stratagene, Heidelberg, Germany). To verify the correct sequence, both strands were sequenced. Subsequently, the p68 RNA helicase cDNA was subcloned into the pcDNA3.1 (+) expression vector (Promega, Mannheim, Germany). HaCaT keratinocytes were transfected using the pcDNA3.1 (+)-p68 cDNA construct. Stable p68 overexpressing cell lines were obtained by selection using genetin (1 mg/ml) for 6 weeks. Subsequently, clones were isolated, subcultured, and tested for p68 overexpression by immunoblot.
HaCaT control cell lines were generated by stable transfection of the pcDNA3.1(+) vector. Geneticin was from Invitrogen.

Transfection Experiments—For transient transfection experiments, 2 × 10⁵ cells (HaCaT, human embryonal kidney cells (HEK)) were plated and grown for 24 h to reach 50–80% confluency. The DNA (5 µg) or small interfering RNA (siRNA, 1 µg) was delivered into the HEK cells using Lipofectamine 2000 (Invitrogen). Transfection of cells was assessed by RNase protection assay. 1000 counts of the hybridization probe was used as a size marker. tRNA was used as a negative control. One representative experiment is shown.

Silencing p68 Expression by siRNA—p68-specific siRNA was generated by Qiagen (Hilden, Germany) using AACCGCAACCAT-TGACGCCAT (nt 152–172, GenBank™ accession number NM_004396) as the cDNA target sequence. The delivered siRNA duplex sequence was formed by the sense 5′-CCGCAACCAT-TGACGCCAT-3′ and antisense 5′-AUGGCCGUAUGGUGCGG-3′ oligonucleotides. As a control, a nonsilencing siRNA duplex with symmetric 2-nt 3′ overhangs of the cDNA target sequence 5′-AATTCTCCGAACGTGTCACG-3′ was used. This sequence only matched with a sequence derived from Thermotoga maritima.

Induction of p68 expression by NO. a, bacterial colonies from an NO-induced differential HaCaT keratinocyte cDNA library were spotted in duplicate onto nylon membranes and screened for regulated sequences by hybridization using α-³²P-radiolabeled cDNAs from nonstimulated control (ctrl) and GSNO-treated HaCaT cells (GSNO) as indicated. The initially identified p68 cDNA clone is indicated by arrows. b, keratinocytes were rendered quiescent and subsequently stimulated with 500 µM GSNO for the indicated times. Induction of p68 mRNA expression in the cells was assessed by RNA protection assay. 1000 counts of the hybridization probe was used as a size marker. tRNA was used as a negative control. One representative experiment is shown. c, quantification of GSNO-induced p68 mRNA (x-fold induction, 5 h after stimulation). **, p < 0.01; *, p < 0.05 (Student's t test) as compared with control. Bars indicate the mean ± S.D. obtained from four (n = 4) independent cell culture experiments. d, immunoblots demonstrating the stimulation of p68 protein expression in quiescent HaCaT cells by the NO donors GSNO (upper panel) and Sp-NO (lower panel). p68-transfected HEK cells (HEK-p68) were used to control the specificity of the anti-p68 antiserum.

Generation of p68-Green Fluorescent Protein (GFP) Fusion Constructs—The human p68 cDNA excluding the stop codon was subcloned from the pBluescript II KS(+) into the HindIII/NheI restriction sites of the pEGFP-N1 vector (Clontech).

Confocal Microscopy—Confocal microscopy was performed using the Zeiss LSM 510 META microscope (Carl Zeiss, Munich, Germany) equipped with a 405 nm laser for 4,6-diamidino-2-phenylindole (emission at 458 nm), a 488 nm laser for GFP/green (emission at 520 nm), and a 543 nm laser for CY3/red (emission at 633 nm). To assess expression of p68-GFP fusion protein, transfected cells were fixed (0.2% formaldehyde in methanol) at −20 °C for at least 1 h. For indirect immunofluorescence, cells were incubated with bovine serum albumin (1 mg/ml in PBS) for 1 h to block nonspecific binding sites. Incubation with the nucleolin-specific antibody (1:100 in PBS/bovine serum albumin) was done at 4 °C overnight. After a final wash with PBS/bovine serum albumin, the FluoroLink™ CyTM3-labeled goat anti-mouse IgG (1:2000) (Amersham Biosciences) was added for 1 h, and DNA was stained with 4,6-diamidino-2-phenylindole (1:1000, 5 min) (Sigma). Preparations were mounted with ProLong Antifade kit (Molecular Probes, Germany).

Proliferation Assay—0.5 × 10⁵ cells (HaCaT keratinocytes or cell lines) per well were seeded into 24-well plates. After reaching 50% confluence, cells were transfected with siRNA specific for p68. Transfected cells or cell lines were starved for 24 h with serum-free Dulbecco’s modified Eagle’s medium. Proliferation of cells was assessed using 1 µCi/ml of [³H]methylthymidine in the presence or absence of serum for 24 h. After 24 h, cells were washed twice with PBS and incubated in 5%
Trichloroacetic acid at 4 °C for 30 min, and the DNA was solubilized in 0.5 M NaOH for 30 min at 37 °C. Finally, \(^{3}\text{H}\) thymidine incorporation was determined.

**Statistical Analysis**—Data are shown as means ± S.D. Data analysis was carried out using the unpaired Student’s \(t\) test with raw data. Statistical comparison between more than two groups was carried out by analysis of variance (ANOVA, Dunnett’s method).

**RESULTS**

**Identification of p68 as a Novel NO-regulated Gene in Keratinocytes**—It is now well established that the short lived radical NO pivotally drives epithelial movements and gene expression during skin repair (22). To gain a deeper insight into NO actions on keratinocyte behavior, we established a differential cDNA library from NO-stimulated HaCaT keratinocytes. Screening of duplicate filters with radioactive-labeled cDNAs from GSNO- and nontreated HaCaT cells identified a bacterial clone that carried a cDNA fragment from a potentially NO-inducible transcript (Fig. 1a). After propagation of the identified clone, isolation of the plasmid, and subsequent sequence analysis, we found a 270-bp cDNA fragment with a 100% nucleotide homology to human p68 DEAD box RNA helicase (p68, GenBank\textsuperscript{TM} accession number NM_004396). As a next step, we had to confirm the potency of NO to induce p68 expression in keratinocytes. To this end, we stimulated quiescent HaCaT keratinocytes using different NO-donating substances. Indeed, we could detect a marked and dose-dependent induction of p68 mRNA expression in the cells (Fig. 1, b and c). Induction of p68 mRNA was followed by a strong increase in p68 protein in NO-induced cells (Fig. 1d). More important, induction of p68 expression was not restricted to GSNO alone, as additional NO-donating agents such as SpNO (Fig. 1d, lower panel) and nitroprusside (data not shown) mediated comparable effects on p68 expression. As a next important step, we tested the specificity of our anti-p68 antiserum, which we had raised in rabbits using a p68-specific peptide sequence. For this reason, we cloned the cDNA of the p68 open reading frame (ORF) into the eucaryotic expression vector pcDNA3.1 (Fig. 2a). First, we performed an \textit{in vitro} transcription/translation assay in the presence of \(^{35}\text{S}\) methionine. Exposure of the corresponding radiolabeled gel revealed the presence of two major bands, from which one clearly represented the expected 68-kDa protein. Moreover, as we transferred the transcription/translation reaction to a polyvinylidene difluoride membrane for immunoblot, we could clearly demonstrate the specificity of the p68 antibody that detects a 68-kDa protein and also the second band, which is most likely to represent an incomplete translation product (Fig. 2b). Additionally, we detected strongly increased levels of p68 in pcDNA3.1-p68-ORF transiently transfected human embryonal kidney (HEK) cells (Fig. 2c) and stably transfected HaCaT keratinocyte cell lines (Fig. 2d).

**p68 Expression Is Induced by Growth Factors, Serum, and Cytokines**—As we are especially interested in keratinocyte movements in the context of skin repair, we now investigated
FIG. 3. Growth factors and serum stimulate p68 expression in keratinocytes. HaCaT keratinocytes were starved for 24 h and subsequently stimulated with 10 ng/ml EGF (a), 10 ng/ml TGF-α (b), 10% FCS (c), or 10 ng/ml KGF (d) for the indicated times. Induction of p68 mRNA expression in the cells was assessed by RNase protection assay. 1000 counts of the hybridization probe was used as a size marker (upper panels). A quantification of p68 mRNA (x-fold induction) is shown in the middle panels. **, $p < 0.01$; *, $p < 0.05$ (ANOVA, Dunnett’s method) as compared with control. Bars indicate the mean ± S.D. obtained from four ($n = 4$) independent cell culture experiments. Immunoblots demonstrating growth factor and serum stimulation of p68 protein expression in HaCaT cells are shown in the lower panels. p68-transfected HEK cells (HEK-p68) were used to control the specificity of the anti-p68 antiserum.
The potency of those mediators to regulate p68 expression that are well established to drive keratinocyte actions during healing in vitro (23, 24). Notably, the potent keratinocyte mitogens EGF (Fig. 3a), TGF-α (Fig. 3b), serum (Fig. 3c), and also EGF (Fig. 3d) were potent inducers of p68 expression in cultured HaCaT cells. Most interestingly, the increase in p68 protein expression was long lasting for all growth factors tested, as we found still elevated p68 levels after 48 h of stimulation (Fig. 3, a–d). Additionally, also a mixture of pro-inflammatory cytokines clearly induced p68 expression in HaCaT keratinocytes (Fig. 4), although p68 protein levels declined earlier when compared with growth factor-stimulated cells. It is known for some time that a mixture of cytokines also induces iNOS expression in keratinocytes (25, 26). To determine a possible role of endogenously produced NO in p68 expression, we treated cells with the cytokines (IL-1β, TNF-α, and IFN-γ) in the presence or absence of the nitric-oxide synthase inhibitor Nω-nitro-L-arginine. However, and most probably because of the delayed appearance of iNOS in the cells after 12 h (25, 26), we could not detect an influence of endogenous NO on p68 expression (data not shown). Taken together, we could determine a series of wound healing associated mediators, in addition to exogenous NO, as potent inducers of p68 expression in keratinocytes.

p68 Protein Expression in Wounded Skin Tissue—Next, we investigated the presence of p68 mRNA and protein expression in normal and wounded murine skin. Moreover, as we had isolated p68 as an NO-inducible gene in keratinocytes, we also analyzed iNOS-deficient (iNOS−/−) mice for p68 expression. We found a constitutive expression of p68 in nonwounded skin in both wild-type and iNOS-deficient animals (Fig. 5, a–c, ctrl skin). However, wounding led to an unexpected expression pattern of p68 in wild-type and iNOS knock-out animals. Although p68 mRNA levels were not significantly altered in healthy animals, we detected a doubling of p68 mRNA expression in wounds from NO-deficient mice that turned out to be significant (Fig. 5a). However, in both experimental setups, p68 protein expression diminished rapidly after wounding in the presence of p68 mRNA (Fig. 5b).

This observation was in clear contrast to the potency of wound-related mediators to stimulate p68 expression in keratinocytes in vitro. Thus, we had to assess the possibility that p68 might be rapidly induced within hours after injury, a time point of induction that could not be detected in 1-day wound samples (Fig. 5b). To this end, we examined p68 expression kinetics within the first 24 h after wounding (Fig. 5c). We observed the overall amount of p68 protein to disappear within hours upon wounding (Fig. 5c). This finding indicated that a rapid increase in overall p68 expression within hours, which could explain our in vitro data in stimulated keratinocytes, did actually not occur. However, we performed a more detailed look on p68 expression in nonwounded skin and within the early wound situation (Fig. 5d). As shown in Fig. 5d (left panel), we found that the epidermal skin compartment nearly completely accounts for the constitutive p68 mRNA expression found in nonwounded skin. As a next step, we separated the wound margin (which predominantly represents epithelial cells) and inner wound (which predominantly represents mesenchymal and immune cells) compartment from freshly isolated 1-day wound tissue to analyze p68 expression. Both compartments were characterized by a strong expression of p68-specific mRNA (Fig. 5d, left panel, inner wd, wd margin). However, it is important to note that the translation of p68 mRNA into protein was clearly restricted to the wound margins (Fig. 5d, right panel). Thus, we could indeed observe spatially restricted but particularly strong expression levels of p68 protein in early wounds (Fig. 5d, right panel), which might be in accordance with our in vitro data.

Most interestingly, p68 protein reappeared at the end of the inflammatory phase of repair in control mice (Fig. 5b, upper panel). Unexpectedly, as we had described p68 to be under a positive regulatory control of exogenously added NO in keratinocytes in vitro, we found a marked re-increase of p68 after injury in NO-deficient mice, which paralleled the increase in p68 mRNA in these animals. Notably, p68 was clearly detectable after 3 and 5 days of repair in iNOS knock-out mice, at time points when p68 was not yet present in those amounts that could be detected by immunoblotting (Fig. 5b).

p68 Protein Is Restrictively Localized in Nuclei of Keratinocytes in Vivo and in Vitro—As we had observed a restricted epithelial expression of p68 mRNA in nonwounded skin, we now determined the localization of p68 protein in normal skin tissue. As shown by immunohistochemistry in Fig. 6a (left panel), p68 protein localization in normal skin tissue was restricted to nuclei of epithelial keratinocytes. Notably, we found about 50% of epithelial keratinocytes to localize p68 within their nuclei. The observed predominant nuclear localization of p68 in nonwounded skin could be confirmed by immunoblotting, where we detected particularly strong p68-specific signals within the isolated nuclear fractions of normal skin tissue (Fig. 6a, left panel).
FIG. 5. **Expression of p68 upon skin wounding.** a, regulation of p68 mRNA expression in C57BLKS (wild-type) and C57BLKS-iNOS⁻/⁻ (iNOS⁻/⁻) mice as assessed by RNase protection assay as indicated. A quantification of p68 mRNA (a-fold induction) is shown. **, p < 0.01; *, p < 0.05 (ANOVA, Dunnett’s method) as compared with control skin. Bars indicate the mean ± S.D. obtained from wounds (n = 48) isolated from animals (n = 12) from three independent animal experiments. b, total protein (100 μg) from lysates of nonwounded and wounded back skin (day 1, 3, 5, 7, and 13 after injury as indicated) isolated from C57BLKS (wt) and C57BLKS-iNOS⁻/⁻ (iNOS⁻/⁻) mice as indicated was analyzed by immunoblot for the presence of p68 protein. c, total protein (100 μg) from lysates of nonwounded and wounded back skin (5, 10, 16, and 24 h after injury as indicated) isolated from C57BLKS mice was analyzed by immunoblot for the presence of p68 protein. Eight wounds (n = 6) from the backs of four animals were excised for every experimental time point and used for protein isolation. Ctrl skin refers to nonwounded skin. p68 protein is indicated by an arrowhead. d, expression of p68 mRNA in nonwounded skin (ctrl skin; epidermis; dermis) and isolated inner wound (inner wd) and wound margin (wd margin) compartments 24 h after injury as assessed by RNase protection assay (left panel). Ctrl skin refers to normal skin tissue including epidermis and dermis. Epidermis and underlying dermis were separated by incubation of tissue in 2 M NaBr for 30 min at 37 °C and subsequently analyzed for p68 mRNA expression by RNase protection assay. 1000 counts of the hybridization probe was used as a size marker. tRNA (20 μg) was used as a negative control. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a loading control. Total protein (100 μg) of isolated inner wound (inner wd) and wound margin (wd margin) compartments 24 h after injury was analyzed for p68-specific protein expression by immunoblot (right panel). Eight wounds (n = 8) from the backs of four animals were excised for every experimental time point and used for protein isolation. p68 protein is indicated by an arrowhead.

6a, right panel). As a next step, we analyzed wound tissue for p68 expression (Fig. 6b). We did so because an absent immunopositive signal for p68 protein in Western blot analysis does not necessarily mean the total absence of the protein at the wound site. Thus, we analyzed 5-day wounds of C57BLKS control mice by immunohistochemistry. It is important to note that we indeed detected immunopositive signals for p68 protein at the wound site. Most interestingly, p68 expression was clearly restricted to keratinocyte nuclei of the developing epithelium (Fig. 6b). Moreover, our p68 antibody recognizes only about one-third of the epithelial nuclei, suggesting that the nuclear presence of p68 might be associated with a distinct phase of the cell cycle (Fig. 6b, arrows). Additionally, it is important to note that the particularly weak p68-specific staining of the nuclei could only be detected when nuclei were not counterstained with hematoxylin (Fig. 6b). Evidently, the weak immunopositive signals in wound sections confirmed the low levels of p68 protein observed by immunoblotting, as the protein indeed was present but clearly detectable only in small amounts at the wound site. However, histological data suggested that we might succeed in detecting p68 protein at early states of healing by immunoblot, when we enrich the wound lysates for nuclei. As shown in Fig. 6b (right panel), we now detected clearly visible amounts of p68 protein in the nuclear fractions of 5- and 7-day wound tissue, strongly confirming our immunohistological findings. Moreover, cultured human HaCaT and murine PAM 212 keratinocyte cell lines also markedly localized p68 protein in the nuclear compartment, as shown by immunoblot (Fig. 6c) as well as transfection experiments with GFP-coupled p68 (Fig. 6d). Moreover, we confirmed the observed nuclear localization of p68 and the potency of NO and TGF-α to induce p68 expression also in cultured murine primary keratinocytes (Fig. 6e).

**Nuclear p68 Localizes in Nucleoli of Keratinocytes—** As a next step, we intended to characterize further the subcellular localization of p68, as we had found the protein predominantly localized in the nuclei of keratinocytes. To this end, we first constructed a p68/GFP fusion protein that was transiently transfected into HaCaT keratinocytes. Again, we could confirm the presence of the transfected p68/GFP fusion protein in nuclear preparations of the cells by using a GFP-specific antibody (Fig. 7a, right panel). Moreover, we assessed the purity of the nuclear fraction by using nucleolin as a marker protein (Fig. 7a, left panel), as nucleolin represents a nonhistone nucleolar protein of eucaryotic cells that is present in abundance at the dense fibrillar and granular regions of the nucleolus (27, 28). Confocal microscopy revealed a strong co-localization of p68/GFP with nucleolin in transfected keratinocytes, and both proteins matched completely in their spatial expression pattern (Fig. 7b). The observed localization strongly suggested the presence of p68 within the nucleoli of the cells, as nucleolin is established to be present in the fibrillar and granular regions of this distinct nuclear subcompartment (27–29).

**p68 Expression Is Functionally Connected to Keratinocyte Proliferation and VEGF Gene Expression—** Finally, we determined whether p68 expression was implicated in the control of...
those important keratinocyte functions that also participate in skin repair. Thus, we investigated the role of p68 protein for keratinocyte proliferation and VEGF gene expression. First, we established HaCaT keratinocyte cell lines that stably overexpressed p68 protein (Fig. 2d). As shown in Fig. 8a (left panel), a constitutive and stable overexpression of p68 led to a strong increase in keratinocyte proliferation rates (+38 ± 6.1% within 24 h) as determined by incorporation of \(^{3}H\)thymidine into the cells. It is important to note that induction of endogenous p68 by serum (Fig. 3c) in control cell lines attenuated the difference in cell proliferation (+20 ± 3.5%) between control and p68-overexpressing cell lines (Fig. 8a, right panel). According to these findings, a down-regulation of endogenous p68 by siRNA techniques in unprocessed HaCaT keratinocytes markedly reduced cellular proliferation rates (Fig. 8b). \(^{3}H\)Thymidine-based proliferation assays of nonstimulated and serum-induced HaCaT cells clearly demonstrated the pivotal role of p68 in keratinocyte proliferation. In serum-free conditions, when p68 is not induced in the cells, a transfection of p68-specific siRNA did not significantly alter the slow keratinocyte proliferation behavior (Fig. 8b, lower right panel). By contrast, transfection of p68-specific siRNA potently shut off serum-induced p68 protein synthesis in the cells (Fig. 8b, upper panel). The observed and potent down-regulation of p68 protein in serum-stimulated cells nearly completely abolished the mitogenic effect that was mediated by serum in the cells (Fig. 8b, lower left panel). Accordingly, immunoblot analysis determined a marked reduction of proliferating cell nuclear antigen as a marker of cell proliferation in siRNA-treated keratinocytes (data not shown).

As DEAD box proteins participate in the regulation of RNA-protein interactions (4), we investigated the possibility that p68 is also functionally implicated in the control of gene expression in keratinocytes. VEGF has been shown to be expressed in wound margin keratinocytes, and its expression undergoes a complex regulation by growth factors, cytokines, and NO (18, 30, 31). For this reason we finally investigated the role p68 in serum- and NO-induced VEGF expression in HaCaT keratinocytes (Fig. 9). Keratinocyte cell lines, which stably overexpressed p68 protein (Fig. 9, p68-ORF 1 and 2), were treated with serum for 5 h. Serum is known to represent an inducer of VEGF expression in keratinocytes (18, 31). Upon serum treatment, the p68 overexpressing cells were characterized by a marked increase in VEGF mRNA levels (Fig. 8a). VEGF has been described as an NO-induced gene in keratinocytes (18), sharing this property with p68 (this study). Thus, it was tempting to speculate that NO-induced p68 might functionally participate in NO-induced VEGF expression. To confirm this hypothesis, we induced treated HaCaT keratinocytes with GSNO in the presence or absence of p68-specific siRNA for 24 h, and we subsequently analyzed the cell culture supernatants by ELISA. As shown in Fig. 9b, down-regulation of endogenous p68 by siRNA transfection significantly reduced the amount of NO-induced VEGF protein (1120 ± 133 pg/ml versus 792 ± 47 pg/ml; \(p < 0.05\)) compared with cells that received the scrambled siRNA oligonucleotides as a control.

**DISCUSSION**

NO is now well established as an important messenger molecule in normal skin biology and also diseased skin (32, 33). Studies on skin tissue have also demonstrated a key function of NO in the process of cutaneous wound healing (22). iNOS-deficient mice suffered from a delay in wound repair (34). Thus, an early induction of iNOS upon wounding has been well documented (35), and inhibition of iNOS enzymatic activity during healing resulted in a markedly reduced re-epithelialization (17). These findings strongly suggested NO to function as a potent mitogenic mediator for skin keratinocytes in vivo. Accordingly, it has been convincingly demonstrated that proliferation of cultured human keratinocytes could be mediated by...
low concentrations of NO (26, 36). However, it is not particularly known how NO exerts its unique actions on keratinocytes. To gain further insight into these mechanisms, we have now established and screened a subtractive keratinocyte cDNA library for NO-regulated genes to characterize new players in NO actions from the cells.

In this study, we have identified p68 DEAD box RNA helicase as a novel NO-induced gene in keratinocytes. Besides the not yet characterized transcripts that have been additionally isolated by using this approach, we focused on p68, as previous work strongly suggested a role of this protein in dynamic cellular movements such as proliferation, transcription, translation, RNA splicing and stability, or ribosome assembly (4). Evidently, we hypothesized that p68 might at least partially contribute to or, moreover, mediate some of the well established actions of NO in keratinocyte biology. NO has been shown to control mRNA levels of a series of wound-related genes in keratinocytes such as VEGF or different chemokines (18, 37, 38). Additionally, we have identified Cu,Zn-SOD to be exclusively induced by NO in keratinocytes. Moreover, we have been able to functionally connect the enzymatic actions of the Cu,Zn-SOD to keratinocyte proliferation. Nevertheless, overexpression of the SOD enzyme did not alter gene expression in the cells (26). Thus, NO actions on keratinocyte gene expression and proliferation appeared to be clearly dissected in molecular terms, as we had not yet identified a regulatory player in NO-controlled gene expression that also participated in the control of keratinocyte proliferation. Regarding published data, it was now reasonable to suggest that p68 might serve a control of both keratinocyte gene expression as well as proliferation.

The situation for p68 actions appeared to be complex with respect to NO. We could convincingly demonstrate the potency of exogenous NO to induce p68 expression in keratinocytes. Nevertheless, although lower amounts of NO (250 μM) were also capable of increasing cellular p68 levels, a potent induction of p68 was only achieved with NO concentrations that have been shown to be cytostatic for cultured keratinocytes (26, 36). By contrast, typical wound-related keratinocyte mitogens appeared to potently trigger an increase in p68 in the cells in vitro. This observation was in accordance with a previous report (15) that demonstrated the induction of p68 in serum-induced proliferating fibroblasts. Moreover, p68 has been associated to hyperproliferative states of epithelial cells, as p68 was described to be overexpressed in nuclei from colorectal adenocarcinomas (16). Although these findings suggested a participation of p68 in cellular proliferation in vitro and in vivo, both studies did not analyze the direct functional contribution of p68 to proliferation under these conditions. However, our results clearly supported the direct contribution of p68 in these processes, as p68 overexpression stimulated and p68 down-regulation attenuated the proliferative behavior of keratinocytes. It is important to note that p68 action on cell proliferation was restricted to mitogen-induced conditions. Thus, we could not find a marked attenuation of keratinocyte proliferation after down-regulation of endogenous p68 under basal, nonstimulated conditions. Evidently, the augmentation of p68 expression seems to represent a pivotal prerequisite to drive serum-induced proliferation in the cells. Thus, it is reasonable to
speculate that serum and, most likely, EGF and KGF as p68-inducing keratinocyte mitogens in vitro might exert parts of their mitogenic potencies on wound keratinocytes by induction of p68.

Indeed, our wound healing studies supported the suggestion that keratinocyte mitogens might overcome the potency of NO to control p68 expression. We found a constitutive expression of p68 in nonwounded skin. Accordingly, a previous study (15) indicated the ubiquitous expression of p68 in newborn and adult mice and rats, respectively. However, although skin tissue was not included, this study suggested that the presence of p68 did not necessarily reflect the extent of cell proliferation in the adult (15). p68 mRNA expression was nearly completely restricted to the epithelial compartment of nonwounded skin, where it is found predominantly in a nuclear localization. As we had recognized the epithelium to account for p68 expression in normal skin, we now subsequently analyzed the epithelial part of early wound tissue. For this reason, we separated the wound margin area from the inner wound tissue. Notably, we found only the wound margin cells to translate p68 protein from high levels of p68-specific mRNA that was present throughout the complete wound tissue. This finding again strongly supports the epithelial expression pattern of p68 protein. Moreover, the particularly strong expression of p68 protein in isolated wound margin lysates in the presence of an overall reduction in p68 protein levels in total wound tissue lysates might indeed argue for a locally restricted induction of p68 expression in wound keratinocytes by wound-related mediators also for the in vivo situation.

It was completely unexpected for us to recognize elevated levels of p68 mRNA and protein in iNOS-deficient animals.
compared with wild-type mice. We found the lowest amounts of p68 in the inflammatory phase of wounding, which is characterized by a dramatic increase in proliferation of keratinocytes located at the margins of the wound. However, it is important to note that p68 had not diminished but could be clearly localized in keratinocyte nuclei of the developing neo-epithelium. Most interestingly, only a defined number of keratinocytes (about 50% in nonwounded skin and about 20–30% in wounds) expressed p68. Thus, it is tempting to speculate that the presence of p68 in keratinocyte nuclei might be associated to a distinct phase of the cell cycle. Consistently, p68 has been described to localize in the nuclei of interphase cells in vitro and in a model of tumor growth.

Evidently, the localization of p68 in interphase cells might provide the explanation for elevated p68 levels observed in NO-deficient healing. INOS-deficient mice were characterized by a marked delay in wound closure (34), and inhibition of iNOS during repair resulted in a nearly complete loss of wound keratinocyte proliferation (17). Thus, the increased levels of p68 in the absence of the keratinocyte mitogen NO strongly argue for high numbers of interphase cells and might reflect the nonproliferative status of keratinocytes in NO-deficient wounds. However, this argument must remain unsolved with respect to cell culture conditions, since, by contrast, we had to recognize that serum-induced keratinocyte proliferation was dependent on the co-induction of p68 (35, 36). Moreover, only a defined number of keratinocytes expressed p68. Thus, it is tempting to speculate that the presence of p68 as an important regulator of keratinocytes movements during skin repair.

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p68 DEAD Box RNA Helicase Expression in Keratinocytes: REGULATION, NUCLEOLAR LOCALIZATION, AND FUNCTIONAL CONNECTION TO PROLIFERATION AND VASCULAR ENDOTHELIAL GROWTH FACTOR GENE EXPRESSION

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