Differential Expression of the Calpactin I Subunits Annexin II and p11 in Cultured Keratinocytes and During Wound Repair

Barbara Munz,* Volker Gerke,† Reinhard Gillitzer,‡ and Sabine Werner*  
*Max-Planck-Institut für Biochemie, Department of Virus Research, Martinsried, Germany; †Universität Münster, Institut für Experimentelle Dermatologie, Münster, Germany; and ‡Universität Würzburg, Klinik und Poliklinik für Haut- und Geschlechtskrankheiten, Würzburg, Germany

Transforming growth factor β1 (TGF-β1) is an important modulator of skin morphogenesis and cutaneous wound repair. To gain insight into the mechanisms of TGF-β1 action in the skin, we used the differential display RT-PCR technique to identify genes that are regulated by this factor in cultured human keratinocytes. We obtained several partial cDNA clones. One of them was identical to the 3'-end of p11, the small and regulatory subunit of the calpactin I complex [(annexin II)p11]. RNase protection and northern blot analysis revealed specific regulation of expression of both subunits of this heterotetrameric protein (p11 and annexin II) by TGF-β1 as well as by other growth factors, although the time course and degree of induction or suppression were different for each gene. Furthermore, we analyzed p11 and annexin II expression in normal and wounded skin.

In cultured keratinocytes and during wound repair, we identified differential display reverse transcription-polymerase chain reaction (DdRT-PCR) as a suitable tool to identify differentially expressed genes.

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The transforming growth factor β (TGF-β) superfamily comprises a broad variety of polypeptides with multiple biologic activities. The family includes TGF-β types 1-5, bone morphogenetic proteins, decapentaplegic, Vg1, Mullerian-inhibiting substance, which consists of two pentaplegic, Vgl, Mullerian-inhibiting substance, 

Furthermore, disturbing the ratio between the three TGF-β isoforms by injection of the ligands or neutralizing antibodies significantly influenced the wound-healing process and the quality of the healed wound. In these experiments, TGF-β1 and -β2 were shown to increase connective tissue deposition and subsequent scarring, whereas TGF-β3 seems to have the opposite effect. Finally, exogenous application of TGF-β1 to a wound enhanced the wound-healing process, particularly in animals with wound-healing defects (for review see Roberts et al., 1996).

To gain insight into the mechanisms of TGF-β1 action in the skin, we have used the differential display reverse transcription-polymerase chain reaction (DdRT-PCR) technique to identify and clone genes regulated by this growth factor in cultured keratinocytes. TGF-β1 was chosen because induction of this isoform during wound repair is particularly early and strong (Frank et al., 1996). We obtained several partial cDNA clones corresponding to differentially expressed genes. One of them was identical to p11, the small and regulatory subunit of the heterotetrameric calpactin I complex, which consists of two p11 and two annexin II subunits (Raynal and Pollard, 1994). Therefore, we analyzed the regulation of both subunits by different growth factors and cytokines in cultured skin.
keratinocytes. Furthermore, we demonstrate differential regulation of p11 and annexin II expression in vivo during wound healing.

MATERIALS AND METHODS

Animals BALB/c mice were obtained from the animal care facility of the Max-Planck-Institute of Biochemistry, Martinsried, and were housed and fed according to federal guidelines.

Wounding and Preparation of Wound Tissue Three independent wound-healing experiments were performed. For each one, 24 BALB/c mice (8-12 wk of age) were anesthetized with a single intraperitoneal injection of avertin. The hair on the animals' backs was cut, and the skin was wiped with 70% ethanol. Six full-thickness excisional wounds (6 mm diameter, 3-4 mm apart) were generated on the back of each animal by excising skin and panniculus carnosus. The wounds were allowed to dry to form a scab. At different time points after injury (24 h to 14 d), animals were sacrificed and wounds were harvested by excising an area of 7-8 mm in diameter. This area includes the scab and the complete epithelial margins. At each time point, the tissue from four animals was combined, immediately frozen in liquid nitrogen, and used for isolation of total cellular RNA (see below). A similar amount of skin from nonwounded animals of the same age served as control. All animal experiments were carried out with permission from the local government of Bavaria.

RNA Isolation, RNAS Protection Assay, and Northern Blotting Isolation of total cellular RNA was performed as described by Chomczynski and Sacchi (1987). RNAS protection assays were carried out as recently described (Werner et al., 1992). For the generation of an 532P-labeled antisense riboprobe, the complete reading frame of human p11 (Saris et al., 1987; Kube et al., 1991) as well as 267-bp fragments of the respective annexin IIIs [mouse: nucleotides 78-344; Saris et al. 1986; human: nucleotides 68-334; Wallner et al., 1986] were amplified by PCR, cloned into the transcription vector pBlueScript KSII(+) (Stratagene, La Jolla, CA), linearized, and used as templates in in vitro transcription reactions. Northern blot analysis was performed as described by Werner et al. (1989): 20 μg of total cellular RNA were separated on a 1% agarose gel containing 5% formaldehyde and blotted onto a Gene Screen Plus Nylon membrane (NEN, Boston, MA). Filters were pre-hybridized in hybridization buffer (50% formamide/5 X sodium citrate chloride/100 μM of Cy5-labeled oligo-mRNA/150 μg per ml tRNA) for 6 h at 42°C. The CDNA fragments of human p11 and annexin II (see above) were labeled by the random primer extension method (Feinberg and Vogelstein, 1983) using the rediprime DNA labeling kit (Amersham, Arlington Heights, IL) and ofP-PdCTP (Amersham). Hybridization was carried out overnight at 42°C in hybridization buffer using 2 X 10⁶ cpm of the labeled probe. The next morning, filters were washed in 0.1% sodium citrate chloride/0.1% sodium dodecyl sulfate at 50°C and analyzed by autoradiography.

DDRT-PCR DDRT-PCR was carried out according to Bauer et al. (1994). Briefly, 300 ng of total cellular RNA were reverse transcribed using 5'-dT(19)CG-3' (Cl) as 5' primer in a total reaction volume of 30 μl. PCR was performed in a total reaction volume of 20 μl using 5 μCi of 32p-dATP, 2.5 mM downstream primer (Cl), 0.5 mM upstream primer (N6: 5'-d(AAACCCTGCCG-3'), and 1 U Taq Polymerase (Perkin-Elmer, Norwalk, CT). Forty cycles were performed (annealing temperature 42°C, elongation period 30 s).

Tissue Culture HaCaT keratinocytes (Boukamp et al., 1988) were grown to confluency in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and were rendered quiescent by serum starvation for 16 h. Cells were stimulated with 1 ng TGF-β1 per ml, 20 μg epidermal growth factor (EGF) per ml, 10 ng keratinocyte growth factor (KGF) per ml, 300 U tumor necrosis factor-α (TNF-α) per ml, or 10% (vol/vol) FBS for various periods (1 to 24 h). Cells were washed twice with PBS and total cellular RNA was isolated as described above. Each experiment was repeated at least twice. Dulbecco's modified Eagle's medium and FBS were purchased from GibCO/BRL; growth factors and cytokines were from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Immunohistochemistry Six-micrometer frozen sections from 5-d murine excisional and from 4-d human incisional skin wounds were fixed with acetone and treated for 3 min at room temperature with 1% H2O2 in phosphate-buffered saline to block endogenous peroxidase activity. They were subsequently incubated overnight at 4°C with a monoclonal anti-annexin II antiserum recognizing human and murine annexin II (Thiel et al., 1991) (1:100 dilution in phosphate-buffered saline, 0.1% bovine serum albumin) or with a monoclonal anti-p11 antiserum recognizing human p11 (Osborn et al., 1988) (1:1000 diluted in phosphate buffered saline, 0.1% bovine serum albumin). The specificity of these antisera had previously been assessed by Western blot analysis. The slides were subsequently stained with the avidin-biotin-peroxidase system (Vector Laboratories, Burlingame, CA) using 3-3′-diaminobenzidine as a chromogenic substrate. After development, they were counterstained with hematoxylin and mounted.

RESULTS

Annexin II Expression Is Induced by TGF-β1 in Cultured Human Keratinocytes To identify novel TGF-β1-regulated genes, we stimulated quiescent human keratinocytes (HaCaT cell line) with 1 ng TGF-β1 per ml (Boehringer) for 1, 2, 4, and 8 h. Total cellular RNA was isolated and reverse transcribed using 5'-dT(19)CG-3' as 3' primer. DDRT-PCR was carried out using the same 5'-primer and 5'-d(AAACCCTGGTC)-3' as a 5'-primer. A 413-bp fragment that was found at significantly higher levels after amplification of RNA from TGF-β1-treated cells compared to control cells was eluted from the gel, reamplified, cloned into pBluescriptKSII(+), and sequenced. Sequence analysis revealed that it was identical to the 3'-end of human p11, the small and regulatory subunit of the calpain I complex (Kube et al., 1991; accession numbers M38591 and M81457 in the EMBL database). To confirm the specific induction of p11 by TGF-β1-treated cells, we analyzed the time course of p11 mRNA expression by RNase protection assay. Because our PCR fragment displayed three point mutations, it was not suitable as a template. Therefore, we amplified the complete p11 open reading frame from a HaCaT cDNA and inserted it into pBluescriptKSII(+). As shown in Fig. 1A,C, a 2-fold increase in p11 expression was observed within 4 h after addition of TGF-β1. Highest levels of p11 mRNA were found 12 h after stimulation with this factor. At that time, p11 expression was 5-fold higher than the control. Twenty-four hours after addition of TGF-β1, p11 mRNA concentration was still above basal levels. To determine expression of the large calpain I subunit annexin II was also enhanced by this growth factor, we amplified a 267-bp annexin II cDNA fragment (nt 68-334) and performed RNase protection analysis. As shown in Fig. 1B, annexin II was also expressed at a comparatively high basal level in quiescent keratinocytes. A comparison of the exposure times of the p11 and annexin II protection assays, however, suggests that the latter is expressed at slightly lower levels in cultured keratinocytes, although, due to differences in probe length and uracil content, the RNase protection technology does not allow absolute quantification. Nevertheless, annexin II expression was also upregulated by TGF-β1, the time-course of induction being similar for both subunits (Fig. 1B,C). These results were reproduced in two independent experiments in which corresponding sets of RNAs were used for assessing p11 and p36 expression.

Expression of Annexin II and p11 Is Differentially Regulated by Serum, EGF, KGF, and TGF-α To analyze regulation of annexin II and p11 expression by other growth factors, quiescent HaCaT cells were stimulated with complete serum and with the keratinocyte mitogens EGF or KGF for 5 h and analyzed for p11 and annexin II mRNA expression by Northern blotting (Fig. 2). Surprisingly, we found significantly different regulation of the two genes: p11 expression was strongly induced 5 h after addition of EGF (6.2-fold), but only slightly (1.7-fold) after serum or KGF treatment (Fig. 2A, upper left panel, and Fig. 2B,C). In a parallel experiment, TGF-β1 increased p11 mRNA levels only approximately 2-fold between 4 and 6 h (Fig. 1). Annexin II expression was also stimulated by EGF, but to a much lesser extent (2-fold), which was comparable to the induction seen after serum treatment. After KGF treatment, annexin II expression was even 60% lower compared to the control (Fig. 2A, lower left panel, and Fig. 2B,C). Maximal repression of annexin II expression by KGF occurred between 5 and 9 h after growth factor treatment (data not shown). In contrast to these growth factors, the pro-inflammatory cytokine TNF-α had no effect on p11 and annexin II expression (Fig. 2A, right panels). These results were reproduced by RNase protection analysis using RNAs from an independent tissue culture experiment.
Increased Annexin II and p11 Expression after Skin Injury

To determine whether regulation of annexin II and p11 expression by TGF-β1 and other growth factors might be of physiologic importance, we analyzed expression of these genes during cutaneous wound healing when the levels of these growth factors are significantly elevated (Werner et al., 1992; Frank et al., 1996). For this purpose, we amplified and cloned the complete cDNA of murine p11 and a 267-bp murine annexin II cDNA fragment and performed RNase protection assays using RNA from normal and wounded mouse skin. As shown in Fig 3, expression of both subunits was increased at day 1 after injury. Expression of p11 and annexin II subsequently declined, although with different kinetics.

Differential Expression of p11 and Annexin II in Normal and Wounded Skin

To localize annexin II- and p11-expressing cells in normal and wounded skin, we first compared the expression levels of the corresponding mRNAs in the dermis and the epidermis of human skin. We found high expression of p11 and annexin II in the dermal and epidermal compartments of murine and human skin (data not shown). Figure 4 shows an immunohistochemical anal-
Annexin II belongs to an expanding protein superfamily, and nine different annexins have been described in mammals. All annexins share as a biochemical hallmark the ability to bind to phospholipid membranes in response to elevated calcium levels (reviewed by Smith and Moss, 1994; Reutelingsperger, 1994).

Although much structural information is available, the functions of the different annexins remain enigmatic (Raynal and Pollard, 1994; Liemann and Lewit-Bentley, 1995). Originally, they had been described as 'lipocortins,' i.e., as inhibitors of phospholipase A₂, which mediates the anti-inflammatory response to glucocorticoids (Browning et al., 1990) and, also corresponding to their Ca²⁺- and phospholipid-binding properties, as anticoagulants and modulators of excocytic processes (Raynal and Pollard, 1994).

Furthermore, they have been implicated in the formation or modulation of ion channels, particularly those permeable to Ca²⁺ (Moss et al., 1995) and the regulation of DNA replication as well as cell growth, proliferation, differentiation (Raynal and Pollard, 1994), and apoptosis (McKanna, 1995). Several annexins appear to bind to proteins of the extracellular matrix such as collagen (Raynal and Pollard, 1994) and tenascin (Chung et al., 1994), thereby potentially modulating cell shape and/or motility.

Annexin II is a member of the family that can exist in two physical states, as a monomeric molecule or in a heterotetrameric complex with the small S100 protein p11 (for review see Gerke, 1992). Annexin II as well as the (annexin II)(p11), heterotetramer binds not only to Ca²⁺ and phospholipid but also to F-actin, and the complex is therefore also known as calpactin I (Raynal and Pollard, 1994). Since expression levels of the two calpactin I subunits, i.e., annexin II and p11, are not always coordinated, the degree of tetramerization varies, ranging from 50 to 100% (for review see Gerke, 1989). Annexins I and II are major substrates for numerous tyrosine kinases; mainly the EGF receptor kinase (annexin I) or pp60v-src (annexin II) for review see Raynal and Pollard, 1994). Consequently, tyrosine-phosphorylated annexins are present at high levels in cells transformed by viruses that encode protein-tyrosine-kinases (Radke and Martin, 1979) or in cells treated with growth factors such as EGF (Hunter and Cooper, 1981) or PDGF (Cooper et al., 1982; Isacke et al., 1986), suggesting that annexins might mediate some cellular responses to these factors.

In addition to inducing annexin II phosphorylation, cellular transformation or stimulation of proliferation by growth factor addition can often elevate expression levels of both subunits of the calpactin I complex. Several experiments have demonstrated that p11 and annexin II mRNA levels are increased in numerous transformed cell types irrespective of the nature of the oncogene product, suggesting that annexin II/p11 might be involved in a key step of cellular transformation. In quiescent fibroblasts, p11 as well as p36 expression can be enhanced by serum as well as by a broad variety of single growth factors, whereby both genes are primary responders (Calabretta et al., 1986; Raynal and Pollard, 1994).

Our studies demonstrate that p11 as well as annexin II expression in keratinocytes is stimulated by TGF-β1, a factor that promotes differentiation of epithelial cells. Moreover, the kinetics of induction is quite similar for both subunits, suggesting a constant degree of complex formation. Most remarkably, expression levels of p11 and annexin II were also increased by EGF, a factor that stimulates keratinocyte proliferation, but EGF influenced p11 more than annexin II expression. Upon treatment with KGF, a factor that also stimulates keratinocyte proliferation, annexin II expression was even moderately decreased, although KGF had a slight stimulatory effect on p11 expression. These data suggest that treatment with keratinocyte mitogens might lead to changes in the degree of heterotetramerization and possibly to an excess of p11.

From this background, it was tempting to analyze whether changes in the p11:annexin II ratio might also be associated with keratinocyte proliferation and differentiation processes in vivo.
that purpose, we analyzed p11 and annexin II expression in human and murine skin. Immunohistochemical studies as well as RNase protection analysis demonstrated that p11 and annexin II are both expressed at high levels in the dermis and epidermis of human skin, but the localization of the proteins was significantly different within the epidermis. Whereas p11 was abundant in all epidermal layers, annexin II expression was high only in the basal and lower suprabasal layers. In the upper layers of the normal and hyper-proliferative epithelium, annexin II protein was present at significantly lower levels or was even absent. These different expression patterns of the two calpain I subunits in the epidermis suggest that the p11:annexin II ratio and therefore the degree of complex formation might be associated with the differentiation status of epidermal keratinocytes. Thus, p11 is unlikely to form complexes with annexin II in the suprabasal layers and in the hyper-proliferative epithelium at the wound edge and therefore might have another function in these cells. In this context, it is interesting to note that the human p11 gene has recently been located within a cluster of epidermal differentiation markers on chromosome 1q21 (Volz et al., 1993).

By contrast, immunohistochemical studies demonstrated the presence of high levels of annexin I in basal and suprabasal layers of human epidermis with modified reactivity patterns in hyper-proliferative lesions such as psoriasis, where it is hardly detectable in epidermal keratinocytes (Raynal and Pollard, 1994; Serres et al., 1994a, 1994b). Correspondingly, expression of this protein seems to be linked to a certain level of keratinocyte differentiation (Bastian et al., 1993). On the subcellular level, annexin I appears to be associated with cytoskeletal elements in keratinocytes of the stratum spinosum (Fava et al., 1993).

No data are available concerning possible annexin functions in the skin. Since we could demonstrate that p11 and annexin II expression is regulated by keratinocyte mitogens as well as by inducers of keratinocyte differentiation in vitro, we speculated that it might be instructive to analyze annexin II/p11 expression under in vivo conditions where expression of all these factors is high, e.g., in the process of cutaneous wound repair (Werner et al., 1992; Frank et al., 1996). Under these conditions, a combined action of these factors on different cells present in the wound might occur.

Indeed, we found strong induction of both calpain I subunits after skin injury, and the highest p11 and annexin II levels were observed 24 h after wounding. This early induction might be a result of the action of serum growth factors such as TGF-β1, which are released in large amounts by platelets upon hemorrhage. Expression of p11 and annexin II subsequently declined, although with different kinetics, leading to a different ratio between p11 and annexin II at later stages of the repair process. This altered ratio might lead to a different degree of complex formation, and this hypothesis is strongly supported by our immunohistochemical studies. In summary, our data suggest that wound-derived growth factors might induce expression of annexin II and p11 after skin injury and possibly modify the ratio between the two calpain I subunits.

Figure 4. Immunohistochemical localization of calpain I subunits in normal and wounded skin. Frozen sections (6 μm) from normal human skin (A,B) and from 4-d-old human incisional wounds (C,D) were incubated with monoclonal antibodies against human p11 (A,C) or annexin II (B,D) and stained with a peroxidase detection system using 3-amino-9-ethylcarbazole as a chromogenic substrate. Slides were counterstained with hematoxylin. Positive cells appear red. D, E, and HE indicate dermis, epidermis, and hyper-proliferative epithelium of the wound. Scale bars, 100 μm.
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