Biochemical and Biophysical Research Communications 372 (2008) 103–107

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Thr308 determines Akt1 nuclear localization in insulin-stimulated keratinocytes

Itamar Goren, Elke Müller, Josef Pfeilschifter, Stefan Frank *

pharmazentrum frankfurt/ZAFES, Institut für Allgemeine Pharmakologie und Toxikologie, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt/M, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 29 April 2008
Available online 12 May 2008

Keywords:
Protein kinase B
Keratinocyte
Kinase activation
Insulin
Wound healing

ABSTRACT

Here, we determined the localization and activation of protein kinase B (Akt) in acute cutaneous wound tissue in mice. Akt1 represented the major Akt isoform that was expressed and activated in wound margin keratinocytes and also in the cultured human keratinocyte line HaCaT. Mutation of Akt1 protein, exchanging the activation-essential Ser473 and Thr308 residues for inactive Ala or phosphorylation-mimicking Asp and Glu residues, revealed that phosphorylation of Ser473 represented an essential prerequisite for auto-phosphorylation of Thr308 within the Akt1 protein in keratinocytes. Moreover, cell culture experiments and transfection studies using Thr308 mutated Akt1 proteins demonstrated that phosphorylation of Akt1 at Thr308 appeared to selectively exclude the active kinase from the nucleus and direct the kinase to the cytoplasmic compartment in keratinocytes upon insulin stimulation. In summary, our data show that phosphorylation of Thr308 during insulin-mediated Akt1 activation is an essential prerequisite to exclude Akt1 from the nuclear compartment.

At present there is growing evidence emphasizing a central role of protein kinase B (Akt) in skin and keratinocyte biology. Three highly conserved isoforms of Akt are known: Akt1, Akt2 and Akt3, all of them targets of active phosphatidylinositol 3-kinase (PI3K) [1]. Enzymatic activation of Akt occurs through phosphorylation of the kinase in its activation loop (Thr308) and carboxy-terminal tail (Ser473), respectively [2]. Activation of the PI3K/Akt signaling pathway controls cell survival in differentiating keratinocytes [3–5]. In addition, Akt1/Akt2 double knock-out mice displayed a translucent skin which could be attributed to a reduced proliferation of the basal keratinocytes [6]. These observations were supported by data showing that proliferating human primary keratinocytes expressed only Akt1 and Akt2 isoforms, from which only the targeted reduction of Akt1 was functionally connected to the induction of keratinocyte cell death and the disruption of an organized expression of differentiation markers in organotypic skin cultures [4]. These *in vitro* findings, suggesting Akt1 to promote keratinocyte survival, might find their *in vivo* counterparts during skin repair. Epithelial healing is driven by the response of wound margin keratinocytes toward a diverse multitude of protein-type mediators [7], most of them known to be potent activators of Akt [8]. Among these factors, the severe insulin resistance of chronic wound tissue of diabetic mice particularly suggested the Akt activating insulin [9] to participate in impaired healing [10]. In this study, we observed the Akt1 isoform to be activated in keratinocytes upon skin wounding and in cultured keratinocytes upon insulin stimulation. Moreover, we determined activation of Akt1 at Thr308 to be essential for auto-phosphorylation and cytoplasmic localization of the stimulated kinase in keratinocytes.

Materials and methods

Animals. Female C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany). At the age of 12 weeks, mice were wounded as described below.

Wounding of mice. Wounding of mice was performed as described previously [11,12]. Briefly, 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. Skin biopsy specimens were obtained from the animals 5 days after injury. All animal experiments were performed according to the guidelines and approval of the local Ethics Animal Review Board.

Cell culture. Quiescent human HaCaT keratinocytes [13] were stimulated with insulin (2 μg/ml) for the indicated periods of time. Insulin was from Roche Biochemicals (Mannheim, Germany).

Preparation of protein lysates and immunoblot analysis. Skin and keratinocyte samples were homogenized as described [10,14]. Cytoplasmic and nuclear fractions were generated according to a published protocol by Schreiber et al. [15]. Specific proteins were detected using antisera directed against Akt1 (Epitomics,

* Corresponding author. Fax: +49 069 6301 7942.
E-mail address: S.Frank@em.uni-frankfurt.de (S. Frank).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.04.185

Hamburg, Germany), total Akt, phospho-Akt (Ser473), phospho-Akt (Thr308) (Cell Signaling, New England Biolabs, Frankfurt, Germany), Flag fusion proteins (Sigma, Taufkirchen, Germany), nucleolin and insulin receptor β-subunit (Santa Cruz, Heidelberg, Germany).

Immunoprecipitation. Two hundred micrograms of wound lysate were incubated overnight with the respective Akt1- (Epitomics), Akt2-, Akt3- (Cell Signaling, New England Biolabs) or Flag (Sigma)-specific antibodies according to instructions of the manufacturer. Immunoprecipitates were isolated using protein G Sepharose (Sigma). Flag-tagged Akt1 was immunoprecipitated using an anti-Flag M2 affinity gel (Sigma) in 500 μl of Flag buffer (25 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1 mM CaCl2, and 1% Triton X-100). Protein G Sepharose- and Flag-bound precipitates were washed in TBST (150 mM NaCl, 10 mM Tris–Cl, pH 8.0, and 0.05% Tween 20) and protein was eluted using Laemmli buffer. Triton X-100 and Tween 20 were obtained from Sigma.

Immunohistochemistry. Complete 5-day wounds were isolated from the back, bisected, and frozen in tissue freezing medium. Six micrometer frozen sections were subsequently analyzed using immunohistochemistry as described [11]. Antisera against phospho-Akt (Ser473) and phospho-Akt (Thr308) (Cell Signaling) were used for immunodetection.

Immunofluorescence. Transfected HaCaT keratinocytes were grown on glass slides and stimulated with insulin (2 μg/ml). Control and stimulated cells were subsequently fixed using methanol/EDTA (0.02% w/v) or paraformaldehyde (2% w/v) for 15 min at −20 °C or room temperature, respectively. Fixed cells were blocked with 5% goat serum diluted in PBS/Triton (0.1% w/v). Antiserum against Flag fusion proteins (Sigma) was incubated for 1 h at room temperature. The fluorescence-coupled secondary antibody Alexa Fluor 488 (Molecular Probes, Leiden, The Netherlands) was diluted 1:250 in 5% goat serum/PBS and incubated in the dark for 30 min. Nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI) (Sigma).

Generation of mutated and Akt1-Flag fusion constructs. A full length human Akt1 cDNA was amplified from total HaCaT keratinocyte cDNA using Pfu polymerase (Promega, Mannheim, Germany) and 5′-GATAGAATTCGGGCACCATGAGCGACG-3′ and 5′-CTATGGATCCGGCCGTGCTGC TGGC-3′ as primers. Amplicons were cloned into EcoRI/BamHI sites of pCMV-Flag N3 [16]. The pCMV-NE-Akt1-Flag was used as a template for subsequent cloning strategies. Mutation of Akt1 Thr308 and Ser473 residues was performed using the pCMV-NE-Akt1-Flag vector as a template, the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany) and the following primers: 5′-GGT GCC ACC ATG AAG GCC TTT TGC GGC ACAC-3′ (for the Thr308 to Ala mutation; Akt1-T308A), 5′-CGG TGC CAC CAT GAA GGA ATT CTG CGG CAC ACC TGA GTA CC-3′ (for the Thr308 to Glu mutation; Akt1-T308E), 5′-CCC ACT TCC CCC AGT TCG CGT ACT CGG CCA GCA GCA CG-3′ (for the Ser473 to Ala mutation; Akt1-S473A) and 5′-CCC ACT TCC CCC AGT TCG ACT ACT CGG CCA GCA GCA CG-3′ (for the Ser473 to Asp mutation; Akt1-S473D).

Transfection experiments. HaCaT cells were transiently transfected with FuGene™ transfection reagent (Roche Biochemicals, Mannheim, Germany) and the appropriate plasmid DNA according to the instructions of the manufacturer.

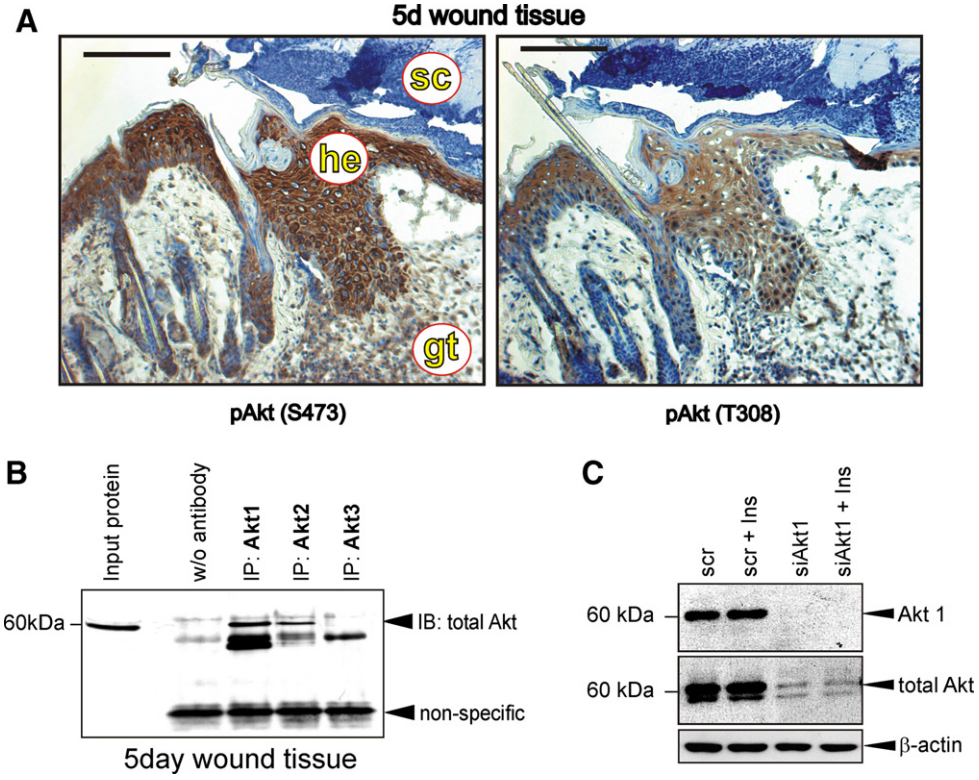

Fig. 1. Localization and isoform expression of Akt in skin wounds and keratinocytes. (A) Frozen serial sections from mouse 5-day wounds were incubated with antibodies directed against phosphorylated Akt (Ser473 or Thr308) as indicated. Nuclei were counterstained with hematoxylin. *gt*, granulation tissue; *he*, hyperproliferative epithelium, *sc*, scab. Bars, 50 μm. (B) Hundred micrograms of total protein from 5-day *wound tissue* were immunoprecipitated using Akt1-, Akt2-, and Akt3-specific antibodies. Immunoprecipitates were analyzed by immunoblot and a total-Akt-specific antibody. Wound lysates were obtained from 8 wounds ($n = 8$) isolated from 4 individual mice ($n = 4$) which have been pooled prior to analysis. (C) Akt1 protein depletion in HaCaT keratinocytes by specific siRNA transfection. Cells were treated with a scrambled (*scr*) or Akt1-specific (*siAkt1*) siRNA in the presence or absence of insulin. Akt1 depletion was controlled using a specific *Akt1* or a *total Akt* recognizing antibody. Loading of the immunoblot was controlled using β-actin.