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Activation of PKB/Akt and p44/42 by mechanical stretch utilizes desmosomal structures and the keratin filament

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ABSTRACT

Background: Mechanical stress is an ubiquitous challenge of human cells with fundamental impact on cell physiology. Previous studies have shown that stretching promotes signalling cascades involved in proliferation and tissue enlargement.
Objective: The present study is dedicated to learn more about cellular structures contributing to perception and signal transmission of cell stretch. In particular, we hypothesized that desmosmal contacts and the adjacent keratin filament build an intercellular matrix providing information about the mechanical load.
Methods: Epidermal cells with different keratin equipment were seeded on flexible silicon dishes and stretched. As read out parameter the activation of PKB/Akt and p44/42 was monitored by Western blotting. Likewise desomosomal contacts were manipulated by depletion or addition of calcium. Moreover, desmoglein 3 and desmocollin 3 were blocked by either specific antibodies or siRNA.
Results: It was found that the omission of calcium from the medium, a necessary cofactor for desmosomal cadherins, inhibited stretch mediated activation of PKB/Akt and p44/42. The relevance of desmosomes in this context was further substantiated by experiments using a desmoglein 3 blocking antibody (AK23) and siRNA against desmocollin 3. Moreover, disruption of the keratin filament by sodium orthovanadate also abrogates PKB/Akt and p44/42 activation in response to stretch. Likewise, KEB-7 keratinocytes harbouring a mutation in the keratin 14 gene and genetically modified keratinocytes devoid of any keratin show an altered signalling after stretch indicating the relevance of the keratin filament in this context.
Conclusion: Besides their important role in cell architecture our results identify desmosomes and keratins as mechanosensing structures.

1. Introduction

It has become apparent that cell geometry and mechanical stress are modulators of basic cellular events including life and death [1,2]. Besides prototypical tissues prone to mechanical stimulation, such as vessels and bones, it turns out that perception of mechanical stimuli is a common feature in most cells playing an important role in maintaining tissue integrity and adaption to different environmental conditions.

In the context of human skin mechanical forces can induce both, differentiation and proliferation, dependent on the quality of stimulation. *In vitro* studies have shown that mechanical pressure promotes differentiation processes [3], while mechanical stretch supports proliferation and anti-apoptosis in human epidermal cells [2,4,5]. These findings fit closely the *in vivo* situation where horny skin is triggered by mechanical pressure as exemplified by the hyperkeratotic finger tips of a violin player. In contrast, mechanical stretch, as present in abdominal stretching during pregnancy, induces skin enlargement. Corroboratory, *in vitro* studies on human skin keratinocytes prove that mechanical stretch induces proliferation-associated signalling cascades

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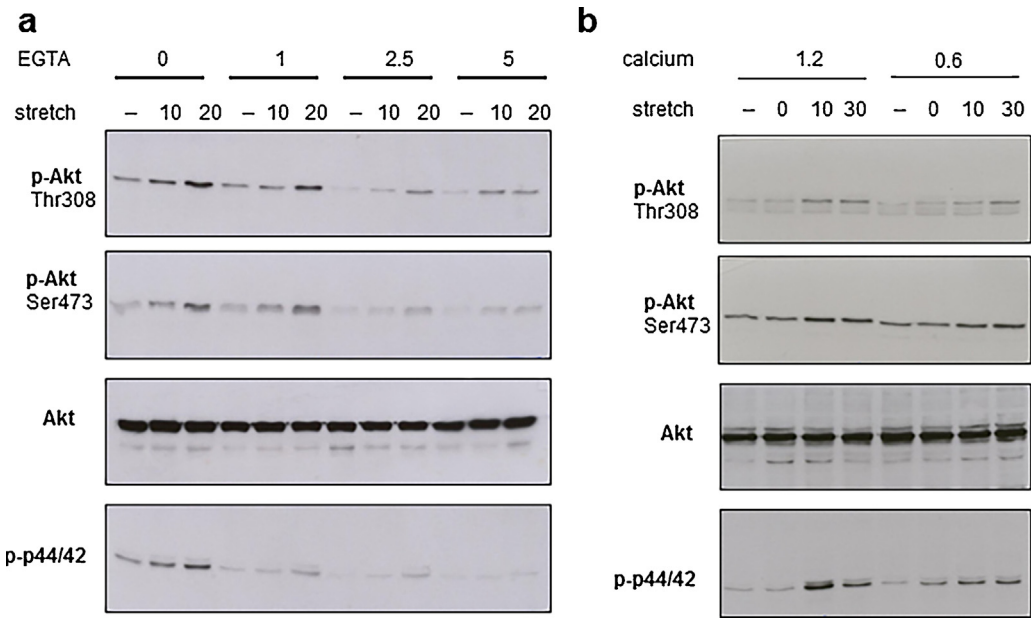

Fig. 1. Low exogenous calcium inhibits stretch induced kinase activation. Serum-starved HaCaT cells were stretched for 5 min, and total protein was prepared after indicated time intervals (min). Protein extracts were subjected to Western blot and tested for phosphorylation of PKB/Akt and p44/42. Equal loading was monitored by using antibodies directed against total PKB/Akt. **(a)** Cells were incubated overnight with 1, 2.5 and 5 mM EGTA, a calcium-chelating compound. **(b)** Quiescent cells were seeded in medium containing 1.2 or 0.6 mM calcium for 24 h, respectively. The blots show representative results (n = 3). p-, phospho.

including activation of mitogen-activated protein kinases (p44/42) and protein kinase B (PKB/Akt). Within human epidermis, keratinocytes have two contact zones both equipped with particular bio-mechanical properties: one is the basement membrane providing an adhesion matrix for basal cells, and the other is represented by intercellular contacts typical for cells residing in the basal layer and in suprabasal layers. Application of an external stretching may be perceived by both contact zones.

In particular, surface receptors of the integrin family, linking extracellular matrix components with intracellular signalling molecules family were discussed to transduce mechanical energy into biological signals [4,6]. Moreover, transactivation of the epidermal growth factor receptor (EGFR) by angiotensin II type 1 receptor (AT-1R) was demonstrated in response to mechanical stretch [2]. Originally, described for bone cells, there are data suggesting a contribution of mechano-sensitive ion channels, including calcium channels [7] and transient receptor potential (TRP) channels [8], being also relevant in epithelial cells. In addition to stretch receptors located on the plasma membrane, it is suspected that mechanical deformation of cytoskeletal elements may be the starting point for some signalling cascades [9]. This assumption is supported by the recent finding that phosphorylation of distinct cytokeratin subsets modulate the mechanical properties of the filament [10]. As cytokeratins link to hemidesmosomes and, particularly to desmosomes, which are specific anchoring junctions imparting calcium-dependent intercellular adhesion by desmogleins (DSG) and desmocollins (DSC), two transmembrane proteins belonging to the cadherin family [11], we hypothesize that the cytokeratin-desmosome complex is important in perception and transduction of mechanical stretch.

2. Materials and methods

2.1. Antibodies and reagents

The pathogenic mouse autoantibody AK23 directed against DSG3 was a kind gift from Masayuki Amagai (Department of Dermatology, Keio University School of Medicine, Tokyo, Japan) [12,13]. AK23, recognizing a calcium-dependent conformational epitope on DSG3, was generated in DSG3-/- mice. After immunization with DSG3, splenocytes of these animals were transferred to DSG3-expressing Rag2-/- recipient mice resulting in stable production of anti-DSG3 IgG autoantibodies. The functionality of AK23 was demonstrated by injection of AK23 to DSG+/+ neonatal animals where microscopic blisters with suprabasilar acantholysis emerge with similarity to pemphigus vulgaris [13]. AK23 was given to the cultures at a final concentration of 1 μg/ml. As control served mouse IgG (Chemicon). For Western blotting the phosphorylation of ERK1/2 was detected using a phospho-specific antibody (Thr202/Tyr204; Cell Signaling Technology, Frankfurt, Germany). Phosphorylation of PKB/Akt (Ser473, Thr308) was detected by phospho-specific antibodies (Cell Signaling Technology). An antibody against total PKB/Akt (Cell Signaling Technologies) served as loading control. The calcium chelating compound ethylene glycol tetraacetic acid (EGTA) was purchased from Applichem (Darmstadt, Germany) and given overnight to the cells at the indicated concentrations. Sodium orthovanadate (OV) was purchased from Sigma Aldrich (Taufkirchen, Germany). OV is a known tyrosine phosphatase inhibitor but recently also described as keratin disrupting compound [14,15]. For this purpose cells were seeded in FCS-free medium and held in complete darkness. At the following day OV was given to the cultures at concentrations ranging from 10 to 100 mM. The effect on the keratin filament was monitored by immunocytochemistry using a pan-cytokeratin antibody (Clone C-11, Sigma-Aldrich). Antibodies against desmosomal plaque proteins were from Cloud Clone (desmoplakin, ABIN1867641) and from Santa Cruz (desmocollin 3, DSC3, sc-81806).

2.2. Cell culture

The spontaneously immortalized human keratinocyte cell line HaCaT (a generous gift from Prof. Fusenig, German Cancer Research Institute, Heidelberg, Germany) was cultured in carbonate buffered Hank's medium with 5% fetal calf serum (FCS) and 1% penicillin/streptomycin solution (P/S) (Biochrom, Berlin, Germany) at 37 °C in a 5% CO_2 atmosphere. For induction of differentiation cells were serum-starved as described [16] and then treated for

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