The MAGUK-family protein CASK is targeted to nuclei of the basal epidermis and controls keratinocyte proliferation

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Summary

The Ca2+/calmodulin-associated Ser/Thr kinase (CASK) binds syndecans and other cell-surface proteins through its PDZ domain and has been implicated in synaptic assembly, epithelial polarity and neuronal gene transcription. We show here that CASK regulates proliferation and adhesion of epidermal keratinocytes. CASK is localised in nuclei of basal keratinocytes in newborn rodent skin and developing hair follicles. Induction of differentiation shifts CASK to the cell membrane, whereas in keratinocytes that have been re-stimulated after serum starvation CASK localisation shifts away from membranes upon entry to S phase. Biochemical fractionation demonstrates that CASK has several subnuclear targets and is found in both nucleoplasmic and nucleoskeletal pools. Knockdown of CASK by RNA interference leads to increased proliferation in cultured keratinocytes. CASK is localised in nuclei of basal keratinocytes and in organotypic skin raft cultures. Accelerated cell cycling in CASK knockdown cells is associated with upregulation of Myc and hyperphosphorylation of Rb. Moreover, CASK-knockdown cells show increased hyperproliferative response to KGF and TGFα, and accelerated attachment and spreading to the collagenous matrix. These functions are reflected in wound healing, where CASK is downregulated in migrating and proliferating wound-edge keratinocytes.

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Key words: CASK, Keratinocytes, Nucleus, Proliferation, Wound healing

Introduction

Epidermal differentiation and wound healing are regulated by several signalling pathways that form an interconnected network (Blanpain et al., 2006; Watt et al., 2006). Growth factors and morphogens that are involved in epidermal biology often require heparan sulfate (HS) as co-receptor for signalling. Examples of HS-dependent signalling proteins include members of the fibroblast growth factor (FGF) family (Rapraeger et al., 1991; Guimond et al., 1993; Loo and Salmivirta, 2002), Wnt proteins (Tsuda et al., 1999; Alexander et al., 2000; Baeg et al., 2001) and members of the transforming growth factor β (TGFβ) and bone morphogenetic protein (BMP) families (Lyon et al., 1997; Rider, 2006).

Syndecans are cell-surface transmembrane HS proteoglycans and are likely to be modulators of the signalling by these growth factors. Syndecans influence cellular signalling by two distinct mechanisms. First, HS chains are required for the formation of signalling complex between several different growth factors and their cognate receptors. Second, the short conserved cytoplasmic domains of syndecans interact with proteins that participate in intracellular signalling and transcriptional control. The best characterised syndecan-interacting proteins are PDZ (PSD-95, discs large, ZO1) domain proteins, such as syndetn (Grootjans et al., 1997) and the Ca2+/calmodulin-associated Ser/Thr kinase (CASK) (Cohen et al., 1998; Hsueh et al., 1998; Hsueh and Sheng, 1999), which both bind to the conserved EFYA sequence in the C-terminus of all syndecans.

CASK belongs to the membrane-associated guanylate kinase (MAGUK) family of scaffolding molecules that are associated with intercellular junctions. Similar to the other MAGUK-family members, CASK consists of an N-terminal PDZ domain, a central SH3 domain and a C-terminal guanylate-kinase homology domain (reviewed by Funke et al., 2005). In addition, CASK is unique in that it also contains an N-terminal Ca2+/calmodulin-dependent protein kinase II (CaMKII) domain (Hata et al., 1996). CASK has been independently identified in Caeorhabditis elegans as LIN-2, a protein required for EGF-receptor localisation during vulval development, and in rat brain as a PDZ-domain protein that binds neurexin and syndecans (Hata et al., 1996; Hsueh et al., 1998). Moreover, the C-terminal guanylate-kinase domain of CASK is a pseudokinase that is involved in re-targeting the protein to the nucleus where it, at least in the case of neuronal cells, interacts with the T-brain (TBR1) transcription factor and the CASK-interacting nucleosome-assembly protein (CINAP), which regulate neuronal gene expression (Hsueh et al., 2000; Wang et al., 2004). Thus, CASK has a dual function as a membrane-associated scaffold protein and as a transcriptional co-regulator.

In the current study, we investigated the role of CASK in the epidermis. It has been previously shown that syndecan 1 and syndecan 4 are involved in epidermal differentiation and repair. Analysis of corneal wound healing in syndecan 1 knockout (KO) mice revealed a failure to upregulate cell proliferation after wounding as well as impairment in corneal cell migration (Stepp et al., 2002). Overexpression of syndecan 1 in the basal layer of
the epidermis by using the keratin 14 promoter leads to temporary hyperproliferation of newborn mouse epidermis but impairs cell proliferation in the newly re-epithelialised epidermis after wounding (Ojeh et al., 2008). Impaired wound healing is also observed in mice that overexpress syndecan 1 systemically in several tissues under the CMV promoter (Elenius et al., 2004). However, an unresolved question is whether CASK, which binds to the cytoplasmic tail of syndecans, influences the regulation of epidermal development and repair.

We report here that CASK regulates proliferation and adhesion of epidermal keratinocytes. CASK is a nuclear protein in developing hair follicles, basal keratinocytes of actively proliferating epidermis of newborn rodents, and cultured human and rodent keratinocytes. In these cells, CASK acts to restrict proliferation as knockdown using small interfering RNA (siRNA) that targets CASK shortens cell-cycle time, amplifies responses to growth factors such as keratinocyte growth factor (KGF) and TGFα, and causes hyperproliferation in organotypic keratinocyte cultures. Knockdown of CASK also accelerates cell adhesion to collagen. These data suggest that CASK is an important regulator of epidermal progenitor cells and participates in the maintenance of epidermal homeostasis.

Results
Nuclear localisation of CASK in developing epidermis and hair follicles
We and others have previously shown that changes in the expression of syndecan 1 influence the regulation of proliferation and differentiation of epidermal keratinocytes (Stepp et al., 2002; Elenius et al., 2004; Ojeh et al., 2008). To investigate whether CASK, a MAGUK-family scaffolding protein that interacts with the conserved cytoplasmic C-termini of syndecan-family members, is involved in keratinocyte proliferation and/or differentiation, we investigated CASK localisation and function in the epidermis and cultured keratinocytes. We first examined CASK expression in developing and adult mouse epidermis by immunofluorescence staining (Fig. 1). In skin of newborn mice, CASK was predominantly found in the nuclei of cells situated in the basal layer, and also in a few cells within the spinous layers. Double staining for the nucleoskeletal protein lamin A and CASK revealed a nuclear colocalisation of both proteins in the basal layer of the epidermis (Fig. 1A, arrows). Some cells that expressed CASK stained also positive for the marker protein of proliferative cells Ki67 – mainly in the basal layer of the epidermis (Fig. 1B, arrows).

Fig. 1. Localisation of CASK expression in skin epidermis and developing hair follicles. (A,B) Immunofluorescence staining of the skin of newborn mice using a rabbit polyclonal anti-CASK antibody (A, B, green channel) and monoclonal JoL-4 antibody against lamin-A (red channel, A') or a monoclonal Ki67 antibody (red channel, B'). Note the predominant nuclear staining for CASK in the basal layer of epidermis. Arrows indicate colocalisation of CASK and lamin A (A') and CASK and Ki67 (B'). (C,D) Immunofluorescence staining of CASK in (C) adult mouse skin and (D) human skin. Dashed lines indicate basement-membrane zone. SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; HF, hair follicle. (E,F) Immunoblotting of whole-cell protein (W), nuclear (N) and cytosolic (C) extracts from mouse primary keratinocytes probed with antibodies against CASK and α-tubulin. (G–K) Strong nuclear CASK expression is seen during rat hair-follicle development. Skin from whisker pads of E18 rats was immunostained for CASK (green) and the adherens-junction marker protein β-catenin (red). (G) Epithelial placode. (H,I) Hair germs. (J) Hair peg. (K) Developing follicle. (L) Western blot analysis of epidermal protein extracts from wild-type (WT), K14-human syndecan-1-transgenic (Tg) and syndecan-1 null (KO) mice probed with antibodies against CASK and involucrin. (M) CASK staining of skin from WT, Tg and KO mice. Notice the increased expression in the skin of Tg mice. Scale bars, 50 μm.
Interestingly, adult mouse epidermis, which is not hyperproliferative similar to the skin of newborn mice, showed a shift in CASK localisation to cell borders within the basal layer of the epidermis (Fig. 1C). A similar pattern of expression was seen in adult human skin, where CASK was detected strongly in the cytoplasm and at the cell-cell junctions within the epidermal basal layer (Fig. 1D).

To further study CASK-localisation patterns in epidermal cells, we examined the localisation of CASK in cultured newborn mouse primary keratinocytes. CASK was distributed in the nucleus, the cytoplasm and at the intercellular junctions of cells (Fig. 1E, arrowheads). To verify this observation, western blot analysis was carried out on nuclear and cytosolic protein fractions obtained from these cells. CASK was present in both the nuclear and cytosolic extracts. The cytoplasmic protein tubulin, used as a control, was present in both the whole-cell protein and cytosolic extracts but was absent from the nuclear extracts (Fig. 1F).

CASK expression during rat hair follicle development was investigated by staining skin sections taken from the whisker pads of rat embryos at gestational age 18 (E18). Strong nuclear localisation of CASK was found in the epidermis, epithelial placode (Fig. 1G), hair germ (Fig. 1H), hair pegs (Fig. 1I,J) and the developing follicle (Fig. 1K). The adherens-junction marker β-catenin was used to delineate the epithelial structures of the developing hair follicles (Fig. 1G-J).

To investigate whether CASK expression levels are regulated by syndecan 1, immunoblotting was performed using epidermal extracts from wild-type mice, transgenic mice expressing human syndecan 1 under the keratin 14 (KRT14, hereafter referred to as K14) promoter (K14 human syndecan 1 transgenic mice) (Ojeh et al., 2008) or syndecan-1–KO animals. CASK expression levels were found to be higher in the epidermal extracts obtained from mice that express human syndecan 1 (Fig. 1L). These transgenic mice overexpress syndecan 1 in the basal epidermis, and the overexpression of CASK in these mice indicates that expression levels of CASK can be regulated by its cognate cell-surface binding partners. The levels of the terminal differentiation marker involucrin, a constituent of cornified envelope within the epidermal cell, remained unchanged in the epidermis of these animals. Immunofluorescence studies also revealed an increase in CASK staining, predominantly in the basal layer but also in the suprabasal layers of the K14 human syndecan 1 transgenic mouse epidermis compared with those of the other animals (Fig. 1M). In addition, increased dermal staining of CASK was seen in the syndecan-1-overexpressing mice, which is in line with the previously reported increased of dermal cellularity in these mice (Ojeh et al., 2008). However, loss of syndecan 1 did not markedly compromise localisation and expression of CASK (Fig. 1M).

Differential status and cell-cycle status regulate the nuclear localisation of CASK

The predominantly nuclear localisation of CASK in basal keratinocytes and during hair-follicle development prompted us to investigate more closely whether the localisation patterns of CASK change during differentiation. We used the human keratinocyte cell line HaCaT, which has retained capacity for differentiation in vitro. A switch from low-Ca²⁺ (0.05–0.1 mM) to high-Ca²⁺ culture conditions (1.2 mM) has been shown to induce differentiation of these cells (Hennings et al., 1980). This was confirmed by epithelial cell-cell adhesion as shown by the accumulation of E-cadherin at cell borders within 24 hours after the Ca²⁺ switch (Fig. 2A), and the expression of involucrin in human primary keratinocytes within 3 days of incubation in the differentiation medium (Fig. 2D). Under low-Ca²⁺ conditions, CASK staining was predominantly nuclear (Fig. 2B) whereas under high-Ca²⁺ conditions, the amount of nuclear CASK reduced concomitantly with the increase in the plasma-membrane-associated pool of CASK (Fig. 2B). This was confirmed by immunoblotting of fractionated cell extracts. Under low-Ca²⁺ conditions, approximately equal proportions of CASK were found in cytoplasmic and nuclear extracts, whereas the proportion of nuclear CASK was markedly reduced in cells grown in differentiation medium (Fig. 2C). These results suggest that CASK is predominantly a nuclear protein in undifferentiated keratinocytes. However, these experiments do not explain the apparent variation in CASK expression within the basal layer of epidermis of newborn mice (Fig. 1) or provide clues to the prominent cell-surface staining in the basal adult human and mouse epidermis. Therefore, we next examined the distribution of CASK in HaCaT cells during the cell cycle.

Serum-starved HaCaT cells in the quiescent state (G0) can be stimulated to re-enter the cell cycle by adding serum. This results in progress through the G1 phase (lasting 0–14 hours), S phase (lasting 14–22 hours with DNA synthesis peaking between 18 and 22 hours after release from quiescence) and, thereafter, the G2-M phase (Geng and Weinberg, 1993). To confirm this, we performed cell-cycle analysis by using flow cytometry on quiescent cells (G0) and stimulated cells again with serum after 6, 12, 22 and 30 hours (Fig. 3A). To see the distribution of CASK during the various phases of the cell cycle, we serum-starved HaCaT cells for 3 days after which we found that >90% cells were in G0, as shown by the FACS analysis (Fig. 3A). During G1 phase, following re-stimulation (6 hours and 12 hours following serum re-stimulation), CASK was detected at sites of cell-cell junctions and in nuclei. After 22 hours, CASK was found predominantly in the nucleus and was lost at cell-cell junctions. The increased presence of CASK in the nucleus correlated with the increased expression of the proliferating-cell marker protein Ki67 (Fig. 3A), as well as with a higher number of cells that had incorporated 5-bromo-2-deoxyuridine (BrdU) – an indication that cells had been dividing during S phase (Fig. 4A). By 30 hours post serum addition, a few mitotic cells were clearly visible (Fig. 3A, arrowheads). This was also confirmed by staining for phosphorylated histone H3 (H3-P) of mitotic cells. At 30 hours mitotic cells were seen that often showed prominent CASK staining in the surrounding cytoplasm, but no colocalisation with H3-P-positive chromatin was observed (Fig. 4B). To analyse CASK levels through the cell cycle, western blotting was performed on whole-cell protein extracts. An increase in the level of CASK expression was seen 6 hours after re-stimulation. This was followed by a decrease in CASK levels at 22 and 30 hours (Fig. 3B) which coincided with the loss of membrane-associated CASK (Fig. 3A and Fig. 4A) and with the appearance of, first, cyclin A at 24 hours and, second, cyclin B by 30 hours (Fig. 3C).

To investigate CASK localisation at specific phases of the cell cycle, we synchronised HaCaT cells by using drugs that block the progression of the cell cycle. Aphidicolin treatment inhibits DNA replication and results in a cell population that is synchronised in G1-S phase, although with the side effect of DNA damage (Kurose et al., 2006). The majority of Aphidicolin-treated HaCaT cells were at the G1-S transition — without any mitotic cells — and all cells that stained positive for the S-phase marker proliferating-cell nuclear antigen (PCNA) showed nuclear localisation of CASK (Fig. 4C). Nocodazole treatment arrests cells at the mitotic spindle
checkpoint and resulted in >70% of HaCaT cells being in G2 and/or M phase (Fig. 4D). In nocodazole-treated cells, strong CASK staining was frequently observed in the cytoplasm surrounding H3-P-stained chromatin. These data are in accordance with the results obtained following serum re-stimulation of quiescent cells.

Subnuclear distribution of CASK reveals nucleoplasmic and nucleoskeletal populations

Since CASK was localised to the nuclei of cells especially under low-Ca\textsuperscript{2+} conditions, we decided to further investigate its subnuclear distribution by examining the solubility properties of CASK in HaCaT cells grown under low or high-Ca\textsuperscript{2+} conditions using a nuclear-matrix-extraction protocol (Dyer et al., 1997; Pekovic et al., 2007) (see Materials and Methods for further details). Fig. 5A shows that, in these cells, CASK was present in all the insoluble pellets after each step of extraction in decreasing amounts. Approximately 40% of CASK first appeared in the soluble fraction after CSK-0.1% Triton X-100 treatment (S2) and ~10% of CASK was present in the soluble fraction after ammonium sulfate extraction (S5). As a control, we used lamin A/C because its solubility properties following sequential extraction has been described in detail (Dyer et al., 1997; Pekovic et al., 2007). As expected, lamin A/C was present in all insoluble pellets after each step of extraction, with only a small amount (~10%) appearing in the soluble fraction after ammonium sulfate extraction.

To investigate changes in solubility properties of nuclear CASK, HaCaT cells were also subjected to nuclear-matrix extraction in situ and examined by immunofluorescence microscopy using antibodies against CASK and lamin A/C. Fig. 5B shows that, without nuclear extraction (stage I), CASK is found in both cell nuclei and cytoplasm. After CSK 0.1% Triton X-100 (stage II) and CSK 0.5% Triton X-100 (stage III) extraction, all cells retained CASK strongly in the nucleus, whereas cytoplasmic CASK was greatly reduced. After DNase I treatment (stage IV) and ammonium sulfate extraction (stage V), CASK was retained in nucleoli (confirmed by anti-nucleolin antibody staining; Fig. 5D), and in the nuclear lamina. Lamin A/C strongly stained the nuclear lamina at all stages of extraction. These results were also similar when a GFP-CASK-transfected HaCaT cells were subjected to the same in situ nuclear-matrix extraction (Fig. 5C,D). These data provide compelling evidence that a population of CASK forms part of the nucleoskeleton in HaCaT keratinocytes.

Knockdown of CASK accelerates cell proliferation and increases growth-factor responses

To investigate the functional role of CASK on cell proliferation, we used siRNA to knockdown CASK in HaCaT cells. Cells were transfected with siRNA targeting CASK or with scrambled siRNA as a control. Western blot analysis revealed an ~80-90%
downregulation of CASK 96 hours post transfection in CASK-siRNA-transfected cells compared with control-siRNA-transfected cells (Fig. 6A). We next measured cell proliferation in an MTT assay using siRNA-transfected HaCaT cells. Preliminary experiments on non-synchronised cells grown with 10% FBS showed a statistically significant increase in HaCaT cell numbers 72 hours after transfection. The same result was obtained with two different CASK-siRNA oligonucleotides (Fig. 6B). To investigate the observed differences in cell number further, we analysed serum-starved and re-stimulated cells at 0 hours (time of siRNA transfection) or 24, 48, 72 and 96 hours following siRNA transfection. We found that knockdown of CASK leads to a significant increase in the growth rate of cells (Fig. 6C). Furthermore, monitoring these cells by phase-contrast microscopy confirmed that CASK-siRNA-transfected cultures had increasingly more cells at 24, 48, 72 and 96 hours after transfection than those
of control-siRNA-transfected cultures (Fig. 6D). Interestingly, the phosphorylated form of the retinoblastoma protein Rb, which controls cell cycle progression, was increased in CASK-siRNA-transfected cells at 72 hours post transfection (Fig. 6E). This was confirmed by immunoblotting using an antibody against all Rb isoforms, which detects both phosphorylated and non-

phosphorylated forms of the protein. Whereas the level of non-phosphorylated Rb remained unchanged, expression of the phosphorylated forms was increased in CASK-siRNA-transfected cells (Fig. 6E).

To confirm these findings on cell proliferation, we performed cell-cycle analysis using flow cytometry on siRNA-transfected

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**Fig. 4.** CASK expression in HaCaT cells following release from cell cycle blockers. (A,B) Serum-starved HaCaT cells were re-stimulated to enter the cell cycle by adding serum. (A) Cells were treated with 10 μM BRDU for 45 minutes prior to staining with rabbit polyclonal anti-CASK antibody (green channel) and mouse monoclonal anti-BrdU antibody (red channel) at 12 and 22 hours. (B) Cells were co-stained with anti-CASK antibody (ZYMED; green channel) and mouse monoclonal anti-H3-P antibody for mitotic cells (red channel) at 22 and 30 hours. (C) Cells synchronised with Aphidicolin were stained with a rabbit polyclonal anti-CASK antibody (ZYMED; green channel) and mouse monoclonal anti-PCNA antibody (red channel). Corresponding cell-cycle analysis is shown in the graph at right. (D) Cells were synchronised with nocodazole prior to staining with a rabbit polyclonal anti-CASK antibody (ZYMED; green channel) and a mouse monoclonal anti-H3 antibody (red channel). Corresponding cell cycle analysis is shown at right. Scale bars, 50 μm.
cells. Serum-starved, quiescent cells were transfected with siRNA, re-stimulated by addition of 10% FBS and harvested at 0 and 60 hours post transfection for FACS analysis. Flow cytometric DNA analysis of the cell cycle revealed that CASK knockdown resulted in a large increase of cells in S phase (36.3%) and G2-M phases (21.3%) and a concomitant reduction of CASK-siRNA-transfected cells in G1 phase (44.8%) compared with control-siRNA-transfected cell populations, of which ~22.8% were in S phase, 13.8% in G2-M phases and 64.5% in G1 phase (Fig. 6F). Likewise, cell-cycle analysis of siRNA-transfected non-synchronised cells indicated that there were more CASK-siRNA-transfected than control-siRNA-transfected cells in S phase 60 hours after transfection (Fig. 6G). These results were also confirmed using cell-cycle analysis based on 5-ethynyl-2'-deoxyuridine (EdU) incorporation (Fig. 7A). Finally, by extrapolating data from the MTT proliferation graph, we calculated that cell-cycle time is approximately 3 hours faster in CASK-knockdown cells. We also investigated the level of apoptosis in both control-siRNA- and CASK-siRNA-transfected cells, which was found to be negligible in both (Fig. 7C), confirming that the differences in cell number counted in MTT proliferation assays were not affected by apoptosis. The expression levels of subunits β1, β4 and α6 of basal integrins were also investigated by flow cytometry and found to be very similar (Fig. 7D) suggesting that the difference in proliferation is not due to changes in basal integrin expression.

To evaluate the mitogenic response of CASK-knockdown cells to growth factors, siRNA-transfected cells were serum starved and left untreated, or were subsequently treated with 10 ng/ml KGF or 10 ng/ml TGFα for 24 hours. Both KGF and TGFα elicited a mitogenic effect on both control-siRNA- and CASK-siRNA-transfected cells. Notably, cell proliferation was enhanced in CASK-knockdown cells compared with control-siRNA-transfected cells after a 24-hour treatment with growth factors (Fig. 7B). Taken together, these data indicate that CASK restricts keratinocyte proliferation and reduces responses to growth factors.

Fig. 5. Biochemical fractionation and in situ nuclear-matrix extraction of HaCaT cells. (A) Western blotting. HaCaT cells grown in low-Ca²⁺ medium were subjected to a sequential extraction by CSK 0.1% Triton X-100 (C/T, 0.1%), CSK 0.5% Triton X-100 (C/T, 0.5%), chromatin digestion by DNase I (DNAse) and a final extraction by 0.25 M ammonium sulfate. Whole-cell protein extracts (P1) were prepared and, following each step, insoluble fractions P2, P3, P4 and P5 were pelleted, and soluble fractions S2, S3, S4 and S5 were retained for immunoblotting with monoclonal anti-CASK antibody and JoL2 antibody to detect lamin A/C. (B) HaCaT cells were grown on glass coverslips in low-Ca²⁺ medium and then subjected to in situ nuclear-matrix extraction using five sequential treatments with CSK buffer only (no extraction; stage I), CSK 0.1% Triton X-100 (stage II), CSK 0.5% Triton X-100 (stage III), DNase I digestion (stage IV) and 0.25 M ammonium sulfate extraction (stage V), and processed for indirect immunofluorescence microscopy using antibodies against CASK (green channel) and lamin A/C (red channel). (C,D) HaCaT cells grown in low-Ca²⁺ DMEM were transfected with a full-length CASK-GFP construct and subjected to in situ nuclear-matrix extraction using five sequential treatments with CSK buffer only (no extraction; stage I), CSK 0.1% Triton X-100 (stage II), CSK 0.5% Triton X-100 (stage III), DNase I digestion (stage IV) and 0.25 M ammonium sulfate extraction (stage V), and processed for indirect immunofluorescence microscopy using antibodies against CASK (green channel) and lamin A/C (red channel). Scale bars, 50 μm (B), 20 μm (C,D).
CASK knockdown increases keratinocyte proliferation in 3D raft cultures

To confirm the effect of CASK knockdown on cell proliferation and to evaluate any effects on differentiation of HaCaT keratinocytes, we seeded CASK-siRNA-transfected and control-siRNA-transfected HaCaT cells on raft cultures and grew them at air-liquid interface for 7 days to allow stratification of keratinocytes. Immunoblotting of protein extracts from the epidermal component of the raft cultures demonstrated that CASK protein levels remained downregulated for the entire culture period (Fig. 8A), which was also confirmed by CASK immunofluorescence staining (Fig. 8B). Levels of the S-phase marker PCNA (Fig. 6A), thickness of the raft epidermis (Fig. 8B), numbers of Ki67 positive nuclei and H3-P-positive mitotic cells (Fig. 8C) were increased. Interestingly, we also observed a marked increase in the protein levels of Myc (Fig. 8A), a known regulator of keratinocyte proliferation (Arnold and Watt, 2001; Waikel et al., 2001; Zanet et al., 2005). The expression of keratins that serve as markers for hyperproliferation, such as keratin 6 (KRT6, hereafter referred to as K6), and basal markers for the epidermis, such as keratins 5 (KRT5, hereafter referred to as K5) and K14, have been shown to be similar in raft cultures as they are in wound healing and hyperproliferative skin diseases (Waseem et al., 1999; Ojeh et al., 2001). As therefore expected, K6, K5 and K14 were similarly expressed in all epidermal layers in both raft cultures (Fig. 8C). Moreover, expression levels of keratinocyte differentiation markers involucrin and filaggrin remained unchanged after CASK ablation (Fig. 8A,C). Even though the most intense involucrin staining appeared in the upper layers in the CASK siRNA raft epidermis compared with control rafts (Fig. 8C), it can be concluded that downregulation of CASK does not prevent keratinocyte differentiation. The basal integrin subunits α6, β1 and β4 were present in the basal layer of both raft cultures, whereas in CASK-siRNA rafts integrin expression was detected also in apical and lateral surfaces of the basal cell and, to a lesser extent, also in suprabasal cells (Fig. 8C).
CASK regulates keratinocyte cell adhesion

Finally, we investigated whether changes in the expression or subcellular localisation of CASK take place during wound healing, where strictly controlled responses to growth factors lead to spatio-temporally controlled proliferation and the migration of keratinocytes. We used immunofluorescence staining to investigate CASK expression levels in three locations of wounded epidermis indicated in the hematoxylin and eosin (H&E) staining of the edge of a punch biopsy wound (Fig. 9A). The epidermal sheet that migrated under the blood clot at the wound site had only a low, mostly cytoplasmic expression of CASK, whereas strong expression was seen in the basal epidermis at the wound edge and in the epidermis further away from the wound site. The downregulation of CASK expression in the migrating epidermal sheet suggested that CASK is involved in keratinocyte migration or adhesion. Attachment of suspended cells to collagen-coated surfaces was accelerated in the absence of CASK (Fig. 9B), which was also reflected by more-pronounced spreading and the formation of focal adhesion sites of the attached cells as shown by vinculin staining (Fig. 9C). Thus, in addition to proliferation, CASK is involved in the regulation of keratinocyte cell-matrix adhesion during wound healing.

Discussion

The aim of this investigation was to gain insights in the role of CASK in the epidermis by studying CASK localisation during skin development and differentiation, and by knocking down CASK in cultured keratinocytes. Our results indicate that CASK restricts keratinocyte proliferation. This conclusion is supported by experiments that compare growth of HaCaT keratinocytes after CASK-siRNA- or control-siRNA transfections, by measuring growth-factor (TGFα and KGF) responses in CASK-knockdown cells and by establishing organotypic 3D raft cultures from CASK-knockdown cells. In all these experimental models, knockdown of CASK resulted in increased keratinocyte proliferation as measured by cell numbers, cell-cycle-dependent analysis of DNA content and expression of cell proliferation markers. Notably, we did not observe any changes in the expression of epidermal differentiation markers after CASK knockdown.

Previous studies have identified three major functions for mammalian CASK. First, CASK participates in the targeting of protein complexes to synapses, as inferred from its interactions with syndecan 2, neurexins and SynCAM (reviewed by Hsueh, 2006). Second, nuclear targeting of CASK and interactions with TBR1 (Hsueh et al., 2000) and CINAP (Wang et al., 2004) modulate transcriptional regulation. Third, CASK contributes to epithelial polarity by interacting with syndecan 1, junctional adhesion molecule (JAM) (Martinez-Estrada et al., 2001) and nephrin (Lehtonen et al., 2005). Intriguingly, both gene targeting (Atasoy et al., 2007) and insertional mutagenesis of the CASK locus (Laverty and Wilson, 1998) lead to cleft palate, implying a role for CASK in embryonic development; but, owing to early neonatal death of
CASK-knockdown mice (Atasoy et al., 2007), the loss-of-function phenotype of CASK is not yet fully characterised. Our results add a novel main function to CASK as a modulator of epidermal proliferation. Notably, mice that lack CASK display increased apoptosis in the thalamus (Atasoy et al., 2007), possibly indicating a different role for CASK in cell survival within the epidermis and brain.

Nuclear localisation of CASK in developing epidermis and cultured keratinocytes

Here, we have shown that, during rat embryonic hair follicle development and in skin of newborn mice, CASK is targeted to the nucleus. In adult skin, however, a shift occurs in the localisation of CASK – to the cytoplasm and cell borders of cells in the epidermis. Interestingly, this shift from nuclear to cytoplasmic CASK also takes

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**Fig. 8.** Knockdown of CASK leads to epidermal hyperproliferation in organotypic cultures. (A) Protein extracts from epidermal tissue were isolated from organotypic cultures, and prepared with control-siRNA- and CASK-siRNA-transfected HaCaT cells after 7 days at the air-liquid interface. Immunoblotting was performed using antibodies against CASK (112 kDa), PCNA (30 kDa), Myc (64 kDa), involucrin (120 kDa) and filaggrin (40 kDa), actin (42 kDa) was used as an internal control. Notice the reduced CASK protein level in CASK-siRNA-transfected epidermal extracts concomitant with the upregulation of PCNA and Myc. (B) H&E histology and immunofluorescence staining for CASK in control and CASK-knockdown HaCaT raft cultures. H&E histology shows a thicker epidermal morphology in the CASK-knockdown 3D cultures, consisting of several layers compared with the control-siRNA-transfected rafts. (C) Expression of keratins, proliferation and differentiation markers, and basal integrins in siRNA-transfected raft cultures. Notice the presence of Ki67-positive cells and H3-P-positive mitotic cells in the CASK-knockdown skin model, in both basal and suprabasal layers of the epidermis. Involutrin and filaggrin are expressed at similar levels in the suprabasal and differentiating layers of the epidermis. CASK knockdown skin model shows integrin expression also in lateral and apical membranes of the basal cells. All protein staining are shown in green and DAPI nuclear staining is shown in blue. Scale bars, 50 μm.
CASK regulates epidermal proliferation

Role of CASK during wound healing

Wound repair is mediated largely by growth factors that are released at the wound site and that have a fundamental role in wound healing, including cellular migration, proliferation and extracellular matrix (ECM) production (reviewed by Werner et al., 2007). Consequently, the role of CASK in regulating keratinocyte proliferation and growth-factor responses is likely to be important in wound healing responses. Interestingly, we found changes in CASK expression at different sites of the epidermis during skin wound healing. CASK expression was downregulated in the migrating epidermal sheet of mouse skin wounds but was upregulated at the wound margin, suggesting that CASK is important for epithelial migration or adhesion. In another study, it
has been shown that during mouse cutaneous repair, the leading cells in the epidermal sheet migrating below the scab were positive for syndecan 1 but the more intense signal was found adjacent to the wound margin at sites of rapid cell proliferation (Elenius et al., 1991). The PDZ domain of CASK is known to bind to the EFYA motif within the C-terminus tail of syndecan (Cohen et al., 1998). Thus, it appears that changes in CASK localisation can be modulated by syndecan expression during wound healing. This was also found to be the case in our transgenic mice studies where over expression of syndecan 1 in mouse epidermis resulted in increased CASK expression. In support of this, subcellular distribution of CASK has been shown to be regulated by the balance of its various binding partners in different subcellular compartments. When syndecan 3 is coexpressed with TBR1 and CASK in COS cells, CASK co-localises with syndecan 3 in the cytoplasm, whereas accumulation of CASK is seen in the nucleus of cultured hippocampal neurons when co-transfected with Tbr1 (Hsueh et al., 2000).

We also observed changes in the rate of adhesion and spreading of CASK siRNA-transfected HaCaT cells compared with control-siRNA-transfected cells when plated on collagen substrates using adhesion assays which measure both cell attachment and spreading. Knockdown of CASK led to faster adhesion and spreading. Similarly, keratinocytes obtained from syndecan-1-null mice were more proliferative and more adherent and migrated more slowly than their wild-type counterparts when seeded onto various ECM substrates, including collagen type I (Stepp et al., 2007). This was largely mediated by α6β4-integrin signalling and TGFβ1 signalling (Stepp et al., 2007). To conclude, we have found a novel role for CASK as a regulator of epidermal proliferation and adhesion that restricts growth-factor responses in keratinocytes. Thus, CASK is likely to be a participant in both physiological and pathological changes such as wound healing or psoriasis.

Materials and Methods

Cell culture

Human normal fibroblasts were isolated from skin and cultured as described previously (Ojeh et al., 2001). Cells were used between passages three and six. Mouse primary keratinocytes were isolated from skin of newborn C57 Black 6 mice and cultured as described previously (Ojeh et al., 2008). The human keratinocyte HaCaT cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For Ca2+-differentiation experiments, HaCaT cells were grown in high-Ca2+ medium [Ca2+-free DMEM supplemented with 1.6 mM Ca2+ and 10% Chelex-treated (Ca2+ free) FBS] or low-Ca2+ medium [Ca2+-free DMEM supplemented with 0.06 mM Ca2+ and 10% chelex-treated FBS]. To induce quiescence through serum starvation, HaCaT cells were switched to DMEM without serum for 3 days. Induction of cell-cycle re-entry was achieved by the addition of 10% FBS to the culture medium. To induce a cell-cycle block at G1-S transition, cells were cultured in FBS-depleted DMEM for 24 hours followed by treatment with 2 ng/ml Aphidicolin (Sigma) in DMEM containing 10% FBS for 24 hours. Synchronisation at mitotic spindle checkpoint was achieved by incubation of the cells with 200 ng/ml nocodazole (Sigma) in DMEM containing 10% FBS without antibiotics and transfected with 100 μg/ml RNase and 50 μg/ml propidium iodide overnight at 4°C. Prior to FACS analysis, cells were centrifuged and diluted in PBS.

Cell-cycle analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson) and data were collected as DNA histograms from 10,000 single-cell events before analysis with the ModFit software using the Dean/Jett-Fox model. In some experiments, cells were labelled with EdU (5-ethyl-2’-deoxyuridine), a thymidine analog that is incorporated into DNA during S phase, similarly to BrdU. 10 μM EdU was added to DMEM and incubated with cells for 45 minutes before harvesting. Staining was performed according to the protocol of the Click-iTTM EdU Alexa-Fluor®-488 cell-proliferation assay kit (Invitrogen). For growth curves, the proliferation of siRNA-transfected HaCaT cells was measured using the colorimetric MTT assay (Promega) at 0 hour (time of siRNA transfection), and 24, 48, 72 and 96 hours post siRNA transfection. Absorbance was measured at 570 nm using a spectrophotometer plate reader (Anthology 1). The effects of growth factors were studied in HaCaT cells transfected with siRNA for 72 hours and then starved for 24 hours in DMEM without serum. KGF and TGFβ (Peprotech) were used at a concentration of 10 ng/ml for a period of 24 hours.

Organotypic 3D skin cultures

Organotypic cultures were prepared as described previously with modifications (Smola et al., 1998). Gels were prepared without fibroblasts or with 5×103 fibroblasts per gel. Following an overnight incubation, 3×105 siRNA-transfected HaCaT cells were seeded on top of each gel and allowed to attach for 24 hours. The cultures were then raised to the air-liquid interface for 7 days. Co-cultures with fibroblasts were embedded in TissueTek OCT compound, snap-frozen and processed for routine haematoxylin and eosin (H&E) histology and immunofluorescence staining. Mono-cultures (gels with HaCaT cells only) were harvested for protein extraction and subjected to western-blot analysis.

Cell adhesion assay

For the measurement of cell attachment and cell spreading, HaCaT cells transfected with siRNA for 96 hours were trypsinised and plated on collagen-coated 24-well plates at densities of 1×105 cells per well. At 30 minutes, 1 hour and 2 hours post seeding, cells were washed three times with PBS (containing 1 mM CaCl2 and 1 mM MgCl2) to remove unbound cells. Attached cells were then processed for immunofluorescence microscopy or subjected to the MTT assay.

Animal immunofluorescence studies

Normal skin tissue from 2-day-old newborn syndecan-1-transgenic mice (Ojeh et al., 2001), syndecan-1-knockout (KO) mice (Stepp et al., 2002) and wild-type C57 Black 6 mice was embedded in TissueTek OCT and snap-frozen. Full-thickness skin wounds on the back of adult C57 Black 6 mice were created as described previously (Ojeh et al., 2008). At day 3 post wounding, normal and wounded skin tissue was embedded in TissueTek OCT and snap-frozen. Cryosections were cut 7-μm thick, and processed for H&E histology and immunofluorescence staining. Mystacial pad regions from E18 gestational age PVG hooded rats were removed, embedded and frozen in TissueTek OCT for immunohistochemistry.

Antibodies and Immunostaining

Immunohistochemistry was performed according to standard laboratory procedures (Long et al., 2006). The primary antibodies used and their dilutions are listed in supplementary material (Table S1). The three different anti-CASK antibodies were used as follows: Antibody from Zymed for human cells and tissues, antibody from Abcam for mouse and rat tissues and primary mouse keratinocytes and the Chemicon antibody for immunoblotting. Secondary antibodies were Alexa-Fluor-488 green (Invitrogen) conjugated anti-rabbit secondary antibody (1:800), Alexa-Fluor-488 green and 594 red (Invitrogen) conjugated anti-mouse secondary antibodies (1:800). DNA of cells was counterstained with DAPI (Sigma). Confocal images of the samples were taken with Zeiss LSM 510 Zeiss or BioRad Radiance 2000 laser scanning confocal microscopes. Composite images were assembled using Adobe Photoshop 7.0 (Adobe® Systems) and LSM510 image browser software (Carl Zeiss).

For nuclear and cytoplasmic protein extracts, cells were incubated in ice-cold hypotonic buffer (10 mM Tris pH 7.4, 10 mM KCl, 3 mM MgCl2, and 0.1% Triton X-100) containing protease-inhibitor cocktail and lysed in a glass-class homogeniser. The cytoplasmic and nuclear fractions were separated by centrifugation at 4000 g for 5 minutes at 4°C. The nuclei-containing pellets were extracted in the same buffer supplemented with 100 U/ml RNase-free DNasel I (Sigma) on ice for 10 minutes. Supernatants were precipitated with ice-cold methanol:acetone (1:1, v/v) at -20°C for 30 minutes, centrifuged at 12,000 g for 10 minutes and washed in PBS. Samples were boiled in 2× Laemmli sample buffer for 5 minutes.

Epidermal protein extracts were obtained from newborn syndecan-1-transgenic mice, syndecan-1-KO mice and wild-type C57 Black 6 mice as described previously (Ojeh et al., 2008). Organotypic cultures were lysed in 2× Laemmli sample buffer. Protein gel electrophoresis and immunoblotting were carried out as described previously (Long et al., 2006).

In situ nuclear matrix extraction and biochemical fractionation of keratinocytes

In situ nuclear-matrix extractions and biochemical fractionation using sequential treatment with detergents, nucleases and salt in the presence of protease-inhibitor
cocktail were performed according to published protocols (Dyer et al., 1997; Pekovic et al., 2007) with minor modifications. Briefly, HaCaT cells grown in low-Ca2+ medium were washed in CSK buffer (10 mM PIPES pH 6.8, 10 mM KCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA pH 8.0) then subjected to extraction in CSK buffer containing 0.1% Triton X-100, CSK buffer containing 0.5% Triton X-100, 100 U/ml RNase-free DNase I (for in situ nuclear-matrix extraction) or 500 U/ml RNase A/DNase I (for biochemical fractionation) in digestion buffer and a final extraction in buffer (10 mM PIPES pH 8.3, 250 mM ammonium sulfate, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA pH 8.0).

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