The Rac activator Tiam1 prevents keratinocyte apoptosis by controlling ROS-mediated ERK phosphorylation

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Summary

Tiam1 is a ubiquitously expressed activator of the small GTPase Rac. Previously, we found that Tiam1 knockout (KO) mice are resistant to DMBA-induced skin tumorigenicity, which correlated with increased apoptosis in keratinocytes of the skin epidermis. Here, we have studied the mechanisms by which Tiam1 protects against apoptosis. We found that Tiam1-KO keratinocytes show increased apoptosis in response to apoptotic stimuli, including growth factor deprivation and heat-shock treatment. Expression of catalytically active Tiam1, but not inactive Tiam1, rescues the apoptosis susceptibility of Tiam1-KO keratinocytes, indicating that this defect is caused by impaired Tiam1-mediated Rac activation. Apoptosis induced by growth factor starvation correlates with impaired ERK phosphorylation in Tiam1-KO keratinocytes. Moreover, Tiam1-KO keratinocytes contain lower levels of intracellular reactive oxygen species (ROS) when compared with wild-type cells. The ROS content of keratinocytes is dependent on both Tiam1 and the activity of NADPH oxidase (Nox), and is required for ERK-mediated survival signaling. Indeed, Tiam1 deficiency or the inhibition of intracellular ROS production blocks ERK phosphorylation and sensitizes wild-type keratinocytes to apoptotic stimuli. Our results indicate that the Rac activator Tiam1 controls the intracellular redox balance by Nox-mediated ROS production, which regulates ERK phosphorylation and the susceptibility of keratinocytes to apoptotic signaling.

Key words: Tiam1, Rac GTPase, Keratinocytes, Apoptosis, Survival signaling, ERK, ROS, Nox

Introduction

In previous studies, we have identified the Tiam1 gene, which encodes an activator of the Rho-like Rac GTPase (Habets et al., 1994; Habets et al., 1995; Michiels et al., 1995). Tiam1 regulates various Rac-mediated signaling pathways, including integrin-mediated cell-matrix adhesions (Hamelers et al., 2005), E-cadherin-mediated cell-cell adhesions (Hordijk et al., 1997; Sander et al., 1998; Malliri et al., 2004), and cell polarity processes (Mertens et al., 2005; Chen and Macara, 2005; Gerard et al., 2007; Pegtel et al., 2007). Tiam1-mediated Rac activation also plays a role in various aspects of tumorigenicity (Malliri et al., 2002; Malliri et al., 2006). Interestingly, the decreased tumor initiation observed in Tiam1-kockout (KO) mice upon DMBA treatment was accompanied by increased apoptosis observed in keratinocytes of the basal layer of the epidermis, suggesting that the DMBA-induced apoptosis prevents the initiation of skin tumors in Tiam1-deficient mice.

Apoptosis is an intrinsic mechanism for the induction of controlled and necessary cell death, for instance during development or viral infection. The prevention of apoptosis by oncogenic agents is a crucial step in the process of tumor initiation. The intrinsic apoptotic pathway is controlled by the family of Bcl2 proteins that regulates mitochondrial integrity and, in particular, cytochrome *c* release. Two Bcl2 subfamilies, consisting of pro-apoptotic proteins that include Bad, Bax and Bak (BAK1), and anti-apoptotic proteins that include Bcl2, Bcl-xL (BCL2L1), and Mlc1, counteract each other in the stimulation or inhibition of mitochondrial cytochrome *c* release (Daniel et al., 2003). The expression and phosphorylation of Bcl2 family proteins is controlled by survival signaling pathways that are predominantly regulated by the PI3K-PKB/Akt (phosphoinositide-3 kinase-protein kinase B) and the MAPK (mitogen activated protein kinases) pathways. Various extracellular stimuli can activate both pathways, including: serum components; growth factors (GFs) such as EGF and IGF1 (Henson and Gibson, 2006; Bernal et al., 2006; Kooijman, 2006); integrins (Cordes, 2006); and small molecule secondary messengers, such as Ca^{2+} or reactive oxygen species (ROS) (Martindale and Holbrook, 2002).

The level of ROS in normal and tumor cells plays an important role in cell survival and apoptosis. Different ROS levels may have opposite effects in the same type of cells. Low ROS concentrations (up to 10-20 μM) are mitogenic and anti-apoptotic (Irani et al., 1997; Arnold et al., 2001; Liu et al., 2005), whereas high ROS levels (above 50-100 μM) induce growth arrest or apoptosis (Stone and Yang, 2006). Physiological levels of ROS are maintained by growth factors, such as PDGF or EGF, which signal primarily via the NADPH oxidase family proteins Nox1-Nox5 (Sundaresan et al., 1995; Bae et al., 1997; Lambeth, 2004). The activity of the Nox1-Nox3 enzymes is regulated by the Rho-like GTPase Rac, and, to a large extent, determines intracellular ROS production (Sauer et al., 2001). Little is known, however, about the activators of Rac that function upstream of Nox.

As the decreased skin tumor initiation observed in Tiam1-KO mice was accompanied by increased apoptosis in keratinocytes of the basal layer of the epidermis (Malliri et al., 2002), we investigated the mechanisms by which Tiam1 could control survival signaling in keratinocytes. To achieve this, we isolated keratinocytes from the skins of wild-type (WT) and Tiam1-KO mice, and studied the

differences in apoptotic and survival signaling in these cells. We found that the susceptibility of keratinocytes for apoptosis induced by GF starvation was dependent on Tiam1/Rac and ERK activation. The ERK-mediated survival pathway was dependent on the presence of intracellular ROS. Tiam1-KO cells are deficient in Rac-dependent activation of Nox and show, therefore, lower ROS production. As ROS stimulates the ERK survival pathway, Tiam1-deficient cells lack this survival-signaling pathway and therefore are more susceptible to apoptotic stimuli.

Results

Tiam1-deficiency increases susceptibility to apoptosis

Tiam1-KO mice are resistant to DMBA/TPA-induced skin tumorigenesis because of increased apoptosis of keratinocytes present in the basal layer of the epidermis (Malliri et al., 2002). To analyze the mechanism by which Tiam1 controls survival and apoptosis in keratinocytes, we used keratinocytes isolated from the skins of newborn WT and Tiam1-KO FVB mice (Mertens et al., 2005). In keratinocyte culture medium, which does not contain serum but contains various keratinocyte growth factor supplements, isolated WT and Tiam1-KO keratinocytes exhibited a similar morphology and a very low percentage of apoptotic cells (Fig. 1A, upper panels). However, when cells were treated with apoptotic stimuli, such as heat shock, profound morphological changes occurred in the cells lacking Tiam1, whereas WT cells showed no effect (Fig. 1A, lower panels). We found that heat-shock treatment or GF deprivation resulted in increased apoptosis in Tiam1-KO cells when compared with WT cells (Fig. 1B,C). The degree of apoptosis was quantified by various means, including DNA content analysis (Fig. 1B) and annexin-V staining (Fig. 1C). We conclude that Tiam1-KO keratinocytes are more susceptible to apoptotic stimuli than WT cells, consistent with the observed increase in apoptosis in the basal layer of the epidermis of Tiam1-KO mice upon DMBA treatment (Malliri et al., 2002). Tiam1 is a specific activator of Rac, and Rac signaling has been implicated in apoptosis resistance (Ruggieri et al., 2001; Le et al., 2005). We therefore analyzed the Rac activity state of WT and Tiam1-KO keratinocytes. Both in normal culture conditions and in the absence of growth factors, Tiam1-KO keratinocytes showed a decreased Rac activity (Fig. 1D), consistent with the function of Tiam1 in the activation of Rac (Michiels et al., 1995).

In order to demonstrate that the increased sensitivity to apoptotic stimuli of Tiam1-KO keratinocytes was due to a lack of Tiam1, we introduced full-length Tiam1 into Tiam1-KO keratinocytes (Fig. 2A). Retroviral transduction of exogenous Tiam1 into Tiam1-KO keratinocytes resulted in a complete rescue of the susceptibility to apoptotic stimuli, such as GF deprivation, as demonstrated by annexin-V staining (Fig. 2B) and aberrant cell morphology (Fig. 2C). Tiam1-KO keratinocytes that expressed exogenous Tiam1 also became insensitive to heat-shock treatment and showed a similar low percentage of apoptotic cells to WT cells (not shown).

We also demonstrated apoptosis biochemically by using caspase-dependent cleavage of specific proteins. Poly ADP-ribose polymerase (PARP) is one of the caspase targets and caspase-mediated proteolysis generates an 89 kDa cleavage fragment of PARP. Additionally, we monitored apoptosis biochemically by the detection of histone-associated DNA fragments in mono- and oligonucleosomes. Both methods were used to further substantiate the function of Tiam1 in the prevention of apoptosis. The downregulation of Tiam1 to approximately 50 percent of the levels found in control cells sensitized WT cells to apoptosis upon GF starvation, as demonstrated by the enhanced PARP cleavage in short hairpin RNA (sh)Tiam1-expressing cells (Fig. 2D). Apoptosis is more pronounced in Tiam1-KO cells, suggesting that apoptosis sensitivity is dependent on the dosage of Tiam1. Indeed, tumor incidence, which is related to apoptosis sensitivity, is also dependent on the dosage of Tiam1, as observed in WT, Tiam1-deficient and Tiam1 heterozygous mice (Malliri et al., 2002; Malliri et al., 2006). This indicates that decreased levels or a lack of Tiam1

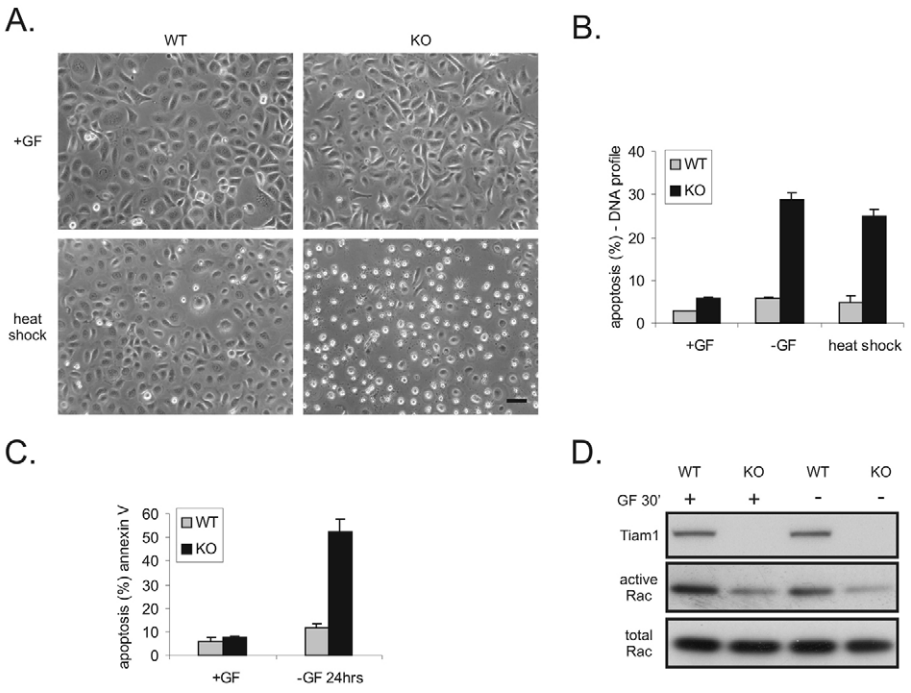

Fig. 1. Tiam1-KO keratinocytes are more susceptible to apoptosis than are wild-type (WT) keratinocytes. (A) Phase-contrast photographs of WT and Tiam1-deficient (KO) keratinocytes cultured on collagen IV-coated tissue culture plates. Cells were grown in normal culture conditions or heat shocked for 90 minutes at 43°C, followed by 6 hours recovery at 37°C. Bar, 50 μm. (B) Quantification of apoptosis induced by GF deprivation (24 hours) or heat-shock treatment in WT and Tiam1-KO keratinocytes. Both adherent and non-adherent cells were collected and subjected to DNA profile analysis. Cells with sub-G1 DNA content were considered to be apoptotic. Error bars represent standard deviation from triplicate samples. (C) Quantification of apoptosis induced by GF deprivation (24 hours) in WT and Tiam1-KO keratinocytes. Both adherent and non-adherent cells were collected, stained with annexin-V and the DNA stain propidium iodide (PI), and analyzed by FACS. Annexin-V-positive cells were considered to be apoptotic. Error bars represent standard deviation from three independent experiments. (D) Rac activity assay performed in lysates from WT and Tiam1-KO keratinocytes. Cells were GF starved for 30 minutes (GF–). Tiam1 and Rac were used as expression and loading controls, respectively. The data presented is a representative example of four independent experiments.

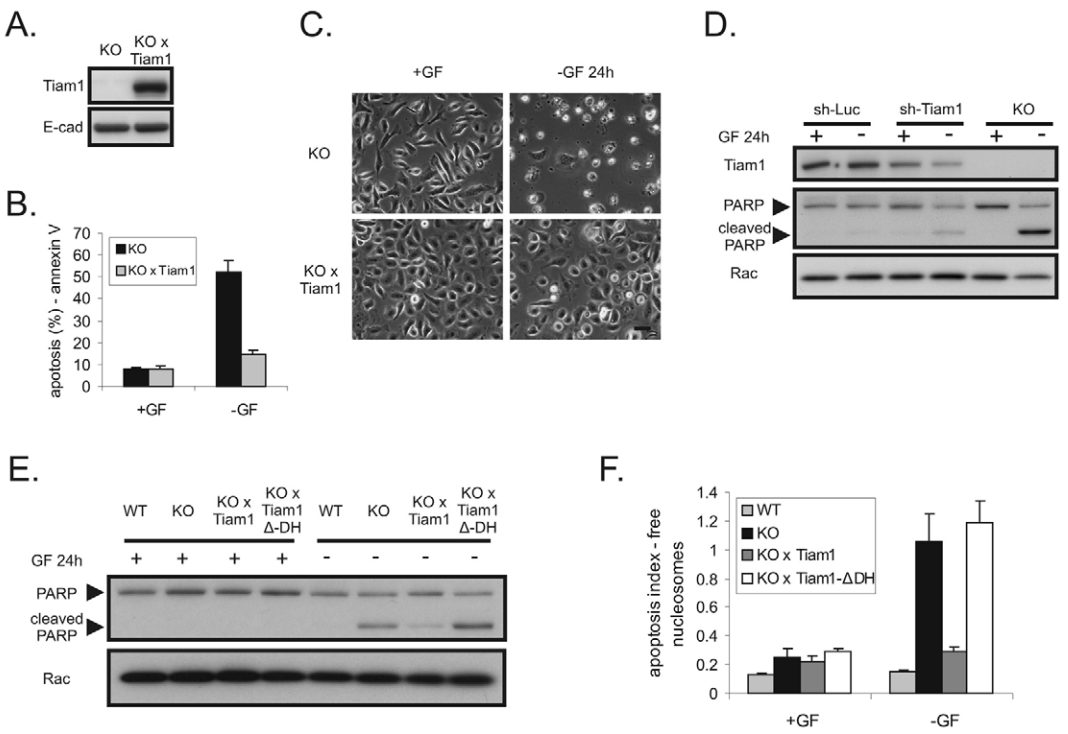

Fig. 2. Expression of Tiam1 rescues the apoptotic sensitivity of Tiam1-KO keratinocytes. (A) Immunoblot showing the expression of exogenous full-length Tiam1 in Tiam1-KO cells. E-cadherin was used as loading control. (B) Quantification of apoptosis upon GF deprivation for 24 hours by annexin-V staining and FACS analysis of Tiam1-KO keratinocytes expressing empty vector or exogenous Tiam1. Error bars represent standard deviation from three independent experiments. (C) Phase-contrast photographs of Tiam1-KO keratinocytes expressing exogenous Tiam1 in GF-containing medium and following GF starvation for 24 hours. Bar, 50 μm. (D) GF starvation (24 hours) induced apoptosis in WT keratinocytes transfected with shRNA constructs and in Tiam1-KO cells. The luciferase targeting sequence was used as a control shRNA (sh-Luc). Tiam1 downregulation was approximately 50%. Apoptosis was monitored by PARP cleavage and was seen only in GF-starved cells expressing Tiam1-specific siRNA or Tiam1-KO cells. (E) Western blot showing keratinocyte apoptosis (PARP cleavage) induced by GF starvation in WT, Tiam1-KO, and Tiam1-KO with exogenous expression of either full-length Tiam1 or an inactive mutant of Tiam1 (Tiam1-ΔDH). Rac expression was used as loading control. (F) Estimation of apoptosis by quantification of free nucleosomes bound to DNA in WT, Tiam1-KO, and Tiam1-KO keratinocytes with exogenous expression of full-length Tiam1 or Tiam1-ΔDH. Only the expression of full-length Tiam1 rescued apoptosis induced in Tiam1-KO keratinocytes by GF starvation. The presented data is a representative example of three independently performed experiments.

are responsible for the increased apoptosis sensitivity found in Tiam1-KO cells.

The exogenous expression of full-length Tiam1 in Tiam1-KO keratinocytes rescued apoptotic sensitivity, as measured by two apoptotic markers, PARP cleavage (Fig. 2E) and free nucleosome appearance (Fig. 2F). However, the expression of Tiam1-ΔDH, which contains a mutation in the Rac activation domain (Michiels et al., 1997), did not rescue apoptosis susceptibility (Fig. 2E,F), indicating that impaired Tiam1-mediated Rac activation is responsible for the increased apoptosis sensitivity of Tiam1-KO cells. From these data, we conclude that the increased susceptibility to apoptosis of Tiam1-deficient cells is due to a lack of Tiam1 and Rac downstream signaling. Apparently, Tiam1-mediated Rac activation is required for cell survival upon apoptotic stimuli.

Tiam1 is required for ERK-mediated survival signaling upon GF starvation

In order to study the mechanisms by which Tiam1 influences apoptosis, we focused on the induction of apoptosis by GF deprivation. The GF supplement (Cascade Biologics) that was added to the keratinocyte medium contains EGF, insulin-like growth factor, hydrocortisone, prostaglandin and transferrin. To determine whether EGF or insulin could rescue the apoptotic effect of GF deprivation, we analyzed apoptosis in keratinocytes grown for 24 hours in medium with and without the complete growth factor supplement, or in medium with EGF or insulin only. As shown in Fig. 3A, both EGF and insulin were able to prevent apoptosis of GF-starved Tiam1-KO cells. This indicates that EGF and insulin GF signaling are normal in Tiam1-deficient cells and that these survival-signaling pathways are not dependent on Tiam1-mediated Rac activation.

To determine the kinetics of apoptosis induced by GF deprivation, we analyzed PARP cleavage in WT and Tiam1-KO keratinocytes at various time points after GF starvation (Fig. 3B, upper panel). Apoptosis in the cell cultures was monitored by the appearance of caspase-truncated PARP. Accumulation of the cleaved PARP product was observed in Tiam1-KO keratinocytes starting from 4 hours after GF deprivation and increased over the next 14 hours (8 and 18 hours). By contrast, WT keratinocytes did not show any PARP cleavage within the observed time frame (Fig. 3B, upper panel), consistent with the absence of apoptosis in WT cells as determined by annexin-V staining (see Figs 1 and 2). Stress- and growth factor-induced ERK activation represent important survival signal pathways in the presence of apoptotic stimuli (Henson and Gibson, 2006). We therefore analyzed the phosphorylation status of ERK upon GF starvation in WT and Tiam1-KO keratinocytes. Phosphorylation of ERK was high in both WT and Tiam1-KO cells in the presence of GFs (compare lanes 1 and 7 in Fig. 3B), confirming our earlier conclusion that GF signaling was normal in

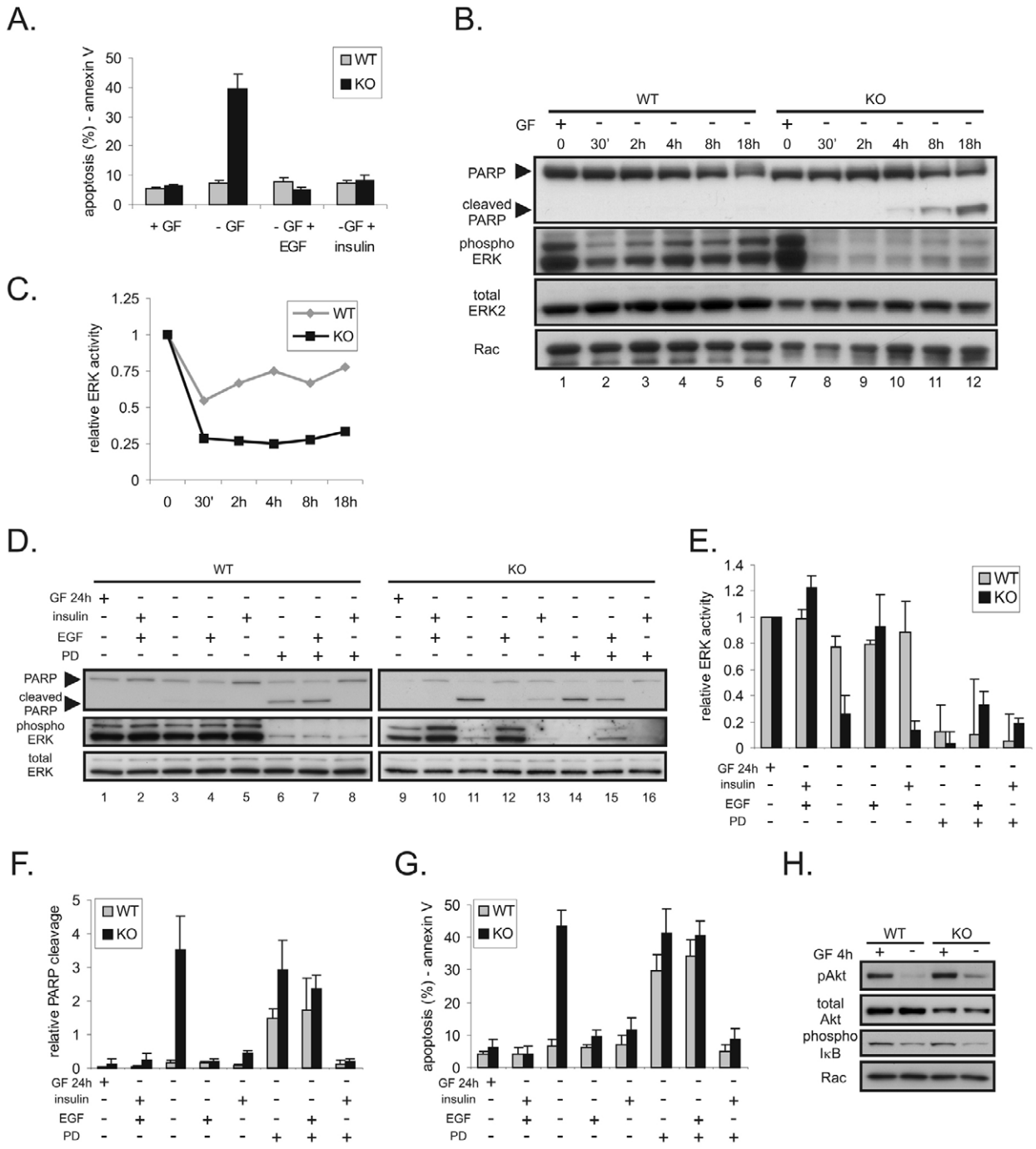

Fig. 3. Tiam1-KO keratinocytes have impaired ERK phosphorylation – a necessary survival signal. (A) Quantification of apoptotic cells by annexin-V staining and subsequent FACS analysis. WT and Tiam1-KO keratinocytes were deprived of complete GF supplement and treated with EGF (20 ng/ml) or insulin (10 μg/ml) as indicated. Replacement of complete GF supplement by EGF or insulin rescued apoptosis. (B) Time-dependent induction of apoptosis and ERK1/2 phosphorylation upon GF starvation. Immunoblot shows PARP cleavage and ERK1/2 phosphorylation; Rac and total ERK are shown as loading controls. (C) Quantification of the data presented in B, normalized to total ERK2. (D) ERK signaling is required for survival upon GF starvation; insulin provides ERK1/2-independent survival signaling. WT and Tiam1-KO keratinocytes were cultured in absence of GF for 24 hours with or without EGF (20 ng/ml), insulin (10 μg/ml) or the MEK inhibitor PD-98059 (10 μM). The immunoblots shown are representative examples of three independent experiments. (E) Quantification of ERK1/2 phosphorylation from three independent experiments with conditions as in D. Values were normalized for total ERK and readouts corresponding to GF-supplemented conditions were set to 1. Error bars represent standard deviation. (F) Quantification of PARP cleaved product from three independent experiments with conditions as in D, values were normalized for total ERK. Error bars represent standard deviation. (G) Quantification of apoptosis induced by GF starvation, by annexin-V staining and FACS analysis. As for the experiment shown in D, WT and Tiam1-KO keratinocytes were cultured in absence of GFs for 24 hours with or without EGF (20 ng/ml), insulin (10 μg/ml) or the MEK inhibitor PD-98059 (10 μM). Error bars represent standard deviation from three independent experiments. (H) Western blot depicting phosphorylation of Akt and IκB in WT and Tiam1-KO keratinocytes in control and GF-deprived conditions (4 hours). Total Akt and Rac are presented as loading controls.

Tiam1-KO cells. Strikingly, upon GF deprivation, an initial drop in ERK phosphorylation was seen, which readily recovered in WT but not in Tiam1-KO keratinocytes (Fig. 3B). Total levels or ERK and Rac1 were measured as loading controls, and data were quantified as shown in Fig. 3C. These findings suggest that Tiam1-deficient cells are sensitive to apoptosis induced by GF deprivation because of impaired GF-independent ERK survival signaling.

In order to substantiate the findings that a lack of ERK activation is causally related to the increased apoptotic sensitivity of Tiam1-KO cells, we treated cells with the chemical ERK inhibitor PD-098059. The PD inhibitor blocks ERK specific kinase (MEK1) and prevents ERK1/2 phosphorylation (Alessi et al., 1995). The PD inhibitor thus prevents survival signals through the ERK pathway (e.g. EGF signaling, stress-induced signaling) but has no effect on survival signals mediated by other pathways, such as the PI3-kinase-Akt pathway (e.g. insulin signaling). As shown in Fig. 3D,G, EGF, or a mixture of insulin and EGF, or total growth factors resulted in ERK activation and prevented apoptosis in both Tiam1-KO and WT cells, as determined by the cleavage of PARP (Fig. 3D, lanes 1-5 and 9-13) and annexin-V staining (Fig. 3G). Both ERK phosphorylation and PARP cleavage have been quantified in Fig. 3E and 3F, respectively. Addition of the PD inhibitor impaired GF-independent ERK activation in WT cells after GF starvation leading to increased apoptosis, as measured by PARP cleavage (Fig. 3D, compare lanes 3 and 6). The PD inhibitor had little effect on Tiam1-KO cells, as no GF-independent ERK activation was seen in these cells (Fig. 3D, compare lanes 11 and 14). PD also inhibited EGF-induced ERK activation, leading to apoptosis in both WT and Tiam1-KO cells cultured in EGF-containing medium (Fig. 3D, lanes 4, 7 and 12, 15). Interestingly, PD inhibited GF-independent ERK activation in insulin-treated WT cells, but did not inhibit insulin-induced survival signals in both WT and Tiam1-KO cells (Fig. 3D, lanes 8 and 16). This is consistent with the findings that insulin-induced survival signaling is mediated through the PI3-kinase/Akt pathway rather than the ERK pathway (Zaka et al., 2005). Insulin, therefore, is able to prevent apoptosis in the presence of the PD inhibitor (Fig. 3D, lanes 8 and 16). From these studies, we conclude that ERK activation is impaired in Tiam1-KO cells upon GF starvation and that this impaired ERK signaling is responsible for the increased susceptibility to apoptosis of Tiam1-deficient cells. Consistent with this, we found no major differences in Akt or IκB phosphorylation between WT and KO cells (Fig. 3H), suggesting that the PI3K-Akt and NFκB survival signaling pathways are not responsible for the differences in apoptosis upon GF starvation.

Tiam1 induces ERK phosphorylation and cell survival by regulating ROS production

ROS, such as H_2O_2, are known to induce ERK phosphorylation and activate the ERK pathway (Guyton et al., 1996). It has been shown that Rac GTPases have an indispensable role in activating ROS-producing enzymes, such as the NADPH oxidase Nox (Miyano et al., 2006; Cheng et al., 2006; Joneson and Bar-Sagi, 1998). As Tiam1 is a specific activator of Rac, we investigated whether Tiam1 could regulate ERK phosphorylation through the production of ROS. Indeed, intracellular ROS levels were significantly lower in Tiam1-KO keratinocytes than in WT cells, as determined by 2′,7′-dichlorofluorescin (DCF) fluorescence measurements in intact cells (Fig. 4A) and cell lysates (Fig. 4B). In WT cells, ROS levels dropped slightly within the first 30 minutes of GF starvation and subsequently recovered to levels found in cells grown in the presence of GFs. These data correlate with the drop and recovery of ERK phosphorylation in WT cells upon GF deprivation (Fig. 3B). Similar results were found by FACS analysis of DCF-stained cells. WT cells contained on average higher ROS levels than did Tiam1-KO cells (Fig. 4C). These data suggest that Tiam1 is involved in ROS production leading to ERK activation. Indeed, the expression of full length Tiam1 in Tiam1-KO keratinocytes (Fig. 4D) increased the level of intracellular ROS in Tiam1-KO cells to that of WT cell levels (Fig. 4E). The expression of Tiam1-ΔDH, which is unable to activate Rac (Michiels et al., 1997), did not increase the levels of ROS (Fig. 4E), indicating that Tiam1-mediated Rac activation is required for the ROS production. Moreover, the downregulation of Tiam1 in WT cells by short hairpin RNA (shRNA) to approximately 50% of the WT levels partly inhibited intracellular ROS production (Fig. 4F). Tiam1 downregulation also resulted in the inhibition of ERK phosphorylation in GF-starved conditions (Fig. 4G). Together, these data suggest that Tiam1-mediated Rac activation is responsible for the control of intracellular ROS and ERK phosphorylation in the absence of GFs.

Tiam1 controls Nox activity that regulates ROS production and ERK phosphorylation

To further substantiate a function of Tiam1 in ROS-mediated ERK phosphorylation and survival signaling, WT keratinocytes were starved in the presence of increasing concentrations of the oxygen scavenger NAC (N-acetylcysteine). Interestingly, treatment of the WT cells with NAC resulted in decreased ERK phosphorylation (Fig. 5A) accompanied by increased sensitivity to apoptotic stimuli, as shown by PARP cleavage (Fig. 5B) and cell morphology changes (Fig. 5C). Note that in the presence of GFs, ROS titration had no effect on ERK phosphorylation or apoptosis (Fig. 5B), indicating that GF-induced survival signaling is mediated by ROS-independent ERK phosphorylation. This is consistent with our conclusion that the GF-induced survival-signaling pathway acts in parallel to the Tiam1/Rac-dependent ERK survival-signaling pathway. In the absence of GFs, ERK phosphorylation is fully dependent on the Tiam1-mediated Rac activation that controls ROS production. Tiam1-KO keratinocytes therefore fail to survive because of impaired ROS-mediated ERK phosphorylation and survival signaling. We wondered what signals could be involved in ERK phosphorylation upon GF starvation. Either cell-cell or cell-matrix interactions could function in ERK-mediated survival signaling in growth factor-deprived conditions. As keratinocytes are cultured in low-Ca^{2+} medium in which no E-cadherin-mediated cell-cell contacts are formed, we concentrated on cell-matrix adhesions. To address this question, attached and suspended WT cells were GF starved. Interestingly we found that ERK activation upon GF starvation is largely impaired in suspended WT cells (Fig. 5D,E), suggesting that integrin-mediated cell-matrix adhesions play a role in ERK-mediated survival signaling.

To investigate the source of ROS that in WT cells supports ERK activation and cell survival, we focused our attention on Nox. Nox enzymes are known for their inducible ROS production and dependency on Rac activity. To test potential Nox involvement in the Tiam1-mediated production of intracellular ROS, we used a Nox-specific inhibitor, diphenyleneiodonium chloride (DPI). The treatment of keratinocytes with DPI resulted in a dramatic inhibition of ROS production in WT cells, whereas ROS production in Tiam1-KO cells was hardly affected (Fig. 6A). This suggests that WT keratinocytes have a much higher fraction of ROS that is produced by Nox and dependent on Tiam1 than do Tiam1-KO cells. Indeed,

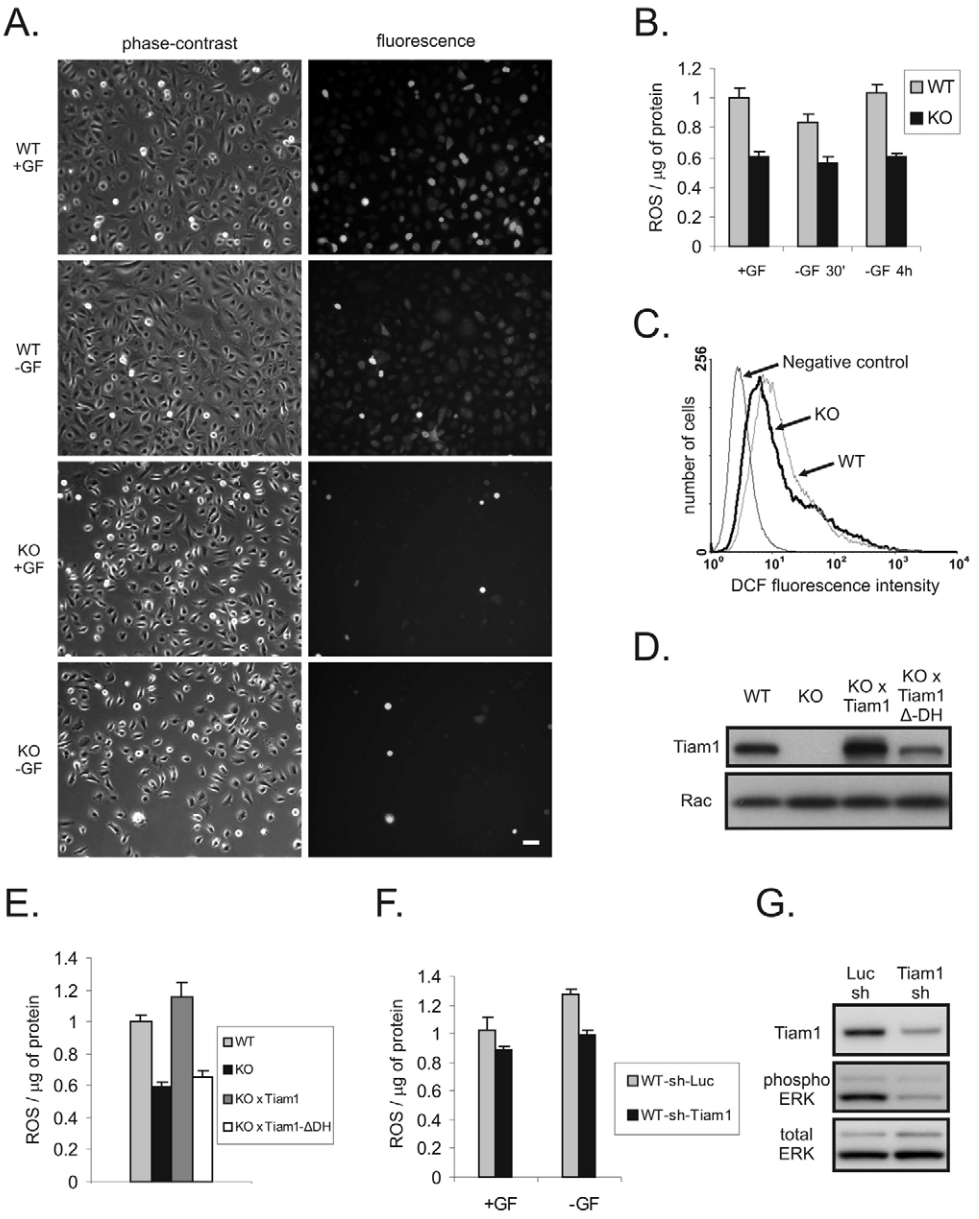

Fig. 4. Tiam1-KO keratinocytes have impaired ROS production. (A) The level of intracellular ROS in WT and Tiam1-KO keratinocytes was visualized by DCF staining. The cells were starved of GFs for 4 hours. Images were taken using an epifluorescence microscope. Bar, 50 µm. (B) Quantification of intracellular ROS in WT and Tiam1-KO keratinocytes upon GF starvation for 30 minutes and 4 hours. Cells were loaded with DCF, lysed and DCF-fluorescence was measured in the lysates. Fluorescence values were normalized for the amount of total protein present in the lysates. The fluorescence value of WT cells was set to 1. Error bars represent standard deviation from triplicate measurements. The result shown is a representative example of four independent experiments. (C) Quantification of ROS levels at a single-cell level in WT and Tiam1-KO keratinocytes by DCF staining and FACS analysis. (D) Western blot showing the exogenous expression of full-length Tiam1 and the deletion mutant of Tiam1 (Tiam1-ΔDH) in Tiam1-KO keratinocytes. Expression of Rac is shown as a loading control. (E) Exogenous expression of full-length Tiam1 but not Tiam1-ΔDH restores ROS production in Tiam1-KO keratinocytes. Intracellular ROS was measured as described in B. (F) Partial downregulation of Tiam1 reduces ROS production in WT keratinocytes, both in the presence and absence of GFs. Intracellular ROS content was measured as described in B. The data is a representative example of three independent experiments. (G) Immunoblot showing Tiam1 expression in WT keratinocytes upon shRNA expression. Partial downregulation of Tiam1 (~50%) inhibits ERK1/2 phosphorylation (in GF-starved conditions) when compared with control cells. Tiam1and total ERK were used as expression and loading controls, respectively.

a general antioxidant, like NAC, decreased intracellular ROS levels similarly in both WT and KO keratinocytes (Fig. 6B). To further substantiate a function of Nox in ROS production, we treated WT and KO cells with another Nox-specific inhibitor (Apocinin). The addition of Apocinin inhibited ROS production in WT cells but not in KO cells (Fig. 6C), similar to the results for DPI (Fig. 6A), further substantiating a function of Nox in Tiam1-mediated ROS production in keratinocytes. To exclude the possibility that a lack of Tiam1 influences the expression of Nox enzyme components, we analyzed the degree of expression of Nox1 and various components of the Nox pathway by western blot. We found no indication that Nox enzymes or Nox components were expressed differently in WT and Tiam1 KO cells (Fig. 6D). Similarly, microarray analyses of WT and Tiam1-KO keratinocytes did not reveal any differences in the expression of Nox and Nox-associated genes between the genotypes (not shown). These data support our conclusion that a lack of Tiam1 decreases ROS production by impaired Rac activation rather than by the impaired expression of ROS-producing enzymes.

The treatment of cells with DPI or NAC had an inhibitory effect on ERK phosphorylation in the absence, but not in the presence, of GFs (Fig. 6E), consistent with our earlier conclusion that GF-induced ERK phosphorylation is independent of ROS (Fig. 5B). Similar results were found upon the induction of apoptosis by heat-shock treatment (Fig. 6F). From these results, we conclude that the susceptibility of Tiam1-KO cells to apoptotic stimuli, such as GF-deprivation and heat-shock treatment, is largely caused by impaired Nox-mediated ROS production, which is required for ERK-mediated survival signaling (see Fig. 6G).

Discussion

In the present study, we show that Tiam1 is involved in cell survival signaling during stress-induced apoptosis. The presence of Tiam1 protects keratinocytes from apoptosis induced by GF starvation and heat-shock treatment. We found that the Tiam1/Rac-mediated survival pathway acts through activation of the ERK pathway. In the absence of GFs, the survival of keratinocytes is completely

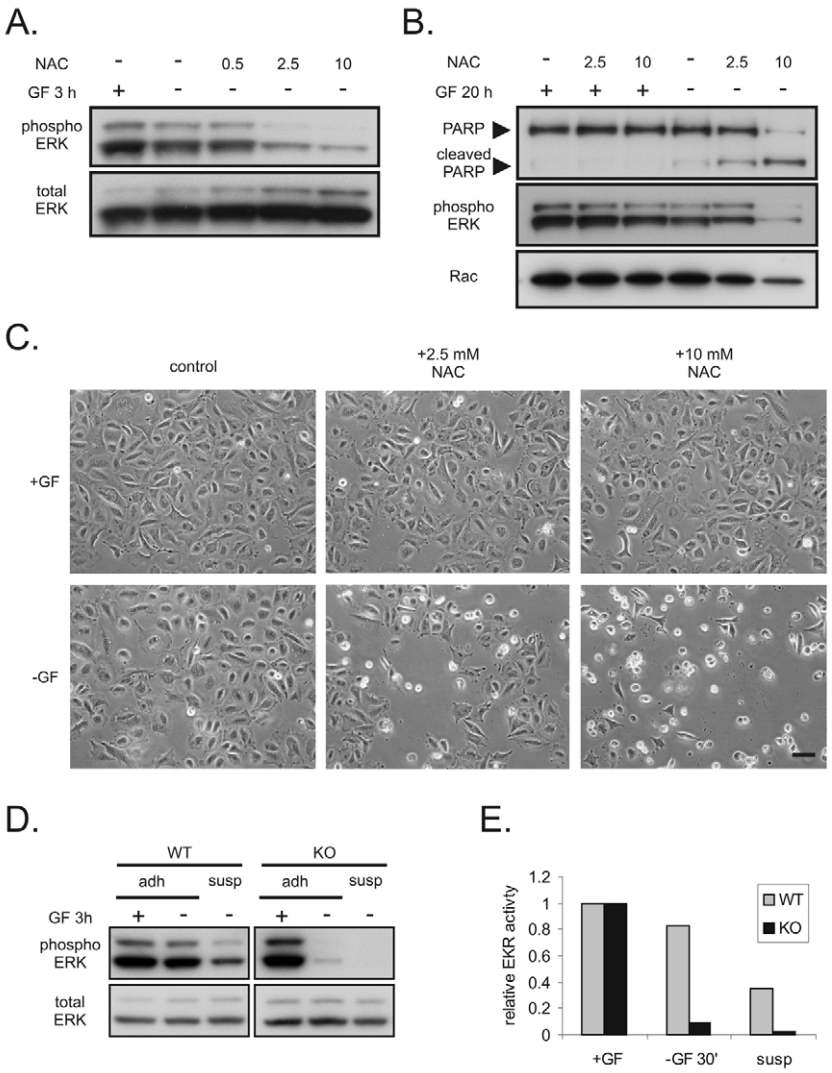

Fig. 5. ROS and cell-matrix adhesions stimulate ERK phosphorylation in the absence of GFs. (A) Western blot depicting that ERK phosphorylation in GF-starved WT keratinocytes is dependent on the presence of ROS and negatively correlates with increasing concentrations of antioxidant (NAC). The cells were starved for 3 hours in the presence of different concentrations of the ROS scavenger NAC. Total ERK expression was used as loading control. (B) Immunoblot showing PARP cleavage and ERK phosphorylation in WT keratinocytes. The cells were cultured in the presence or absence of GFs and NAC, as indicated. PARP cleavage correlates with prolonged (20 hours) GF-starvation combined with NAC treatment. Rac was used as a loading control. NAC was added at the beginning of the experiment. (C) Phase-contrast images of WT keratinocytes subjected to GF starvation and/or NAC treatment. The images correspond to the conditions in B. Bar, 50 µm. The data presented are representative examples of three independent experiments. (D) Western blot showing ERK phosphorylation in WT and Tiam1-KO cells in adhesive control, GF-starved (3 hours) and suspension conditions. Total ERK serves as a loading control. Results are a typical example of three independent experiments. (E) Quantification of ERK activity from D, normalized for total ERK expression; readouts corresponding to GF-supplemented conditions were set to 1.

dependent on Tiam1-mediated ERK activation, which is regulated by intracellular ROS levels and is dependent on cell-matrix adhesions. Tiam1-KO cells have lower intracellular ROS levels than WT cells. The exogenous expression of catalytically active Tiam1 in Tiam1-KO keratinocytes restores ROS levels to those found in WT cells, and thereby reduces the susceptibility to apoptosis. The inhibition of ROS production in WT cells by Nox inhibitors or an antioxidant, such as NAC, reduces both ROS production and ERK phosphorylation, and simultaneously increases the sensitivity to apoptotic signals. From these studies, we conclude that Tiam1 is necessary for the Rac-mediated activation of Nox that leads to the increased ROS production required for ERK-mediated survival signaling following stress signaling, such as GF-deprivation or heat-shock treatment (Fig. 6F).

The activity of Rac has been associated with survival signaling in various model systems. Conditional knockout of both Rac1 and Rac2 in the B-cell lineage results in increased apoptosis during B-cell development (Walmsley et al., 2003). Earlier studies have shown that interleukin-1β-mediated activation of NFκB is dependent on Rac activity and the presence of ROS (Sulciner et al., 1996). Rac is able to prevent anoikis by activation of NFκB downstream of the α6β4 integrin in mammary epithelial cells (Zahir et al., 2003). Rac activity is also required in β1 integrin-mediated survival signaling and the inhibition of anoikis (Hirsch et al., 2002). Interestingly, β1 integrin-mutated mouse embryonic fibroblasts have impaired Rac-dependent ERK nuclear translocation, suggesting an involvement of Rac in ERK signaling and survival (Hirsch et al., 2002). Also, in neuronal cells, Rac seems to play a crucial role in survival signaling, as the inhibition of Rac activity induces apoptosis by stabilizing the Bim protein (Le et al., 2005). Bim is one of the proapoptotic Bcl2 homologs of the BH3 only family and its stability is controlled by ERK phosphorylation (Loucks et al., 2006). ERK phosphorylates Bim on serine 69 and thereby promotes its degradation (Luciano et al., 2003). Furthermore, B cell antigen receptor (BCR)-mediated apoptotic signaling is inhibited by ERK-dependent phosphorylation and degradation of Bim (Craxton et al., 2005). Our data are consistent with reports showing that apoptosis induced by GF starvation is associated with the inhibition of ERK phosphorylation (Manohar et al., 2004). Interestingly, we found that Tiam1-deficient keratinocytes are impaired in Rac activation upon α3β1-mediated adhesion to laminin 5, a cell substrate that is produced by keratinocytes (Hamelers et al., 2005). As survival signaling upon GF starvation is reduced in suspended keratinocytes, it is tempting to speculate that the ERK activation is derived from α3β1 integrin-mediated cell-matrix adhesions. Tiam1 could thus control the Rac-mediated Nox/ROS/ERK pathway downstream of α3β1-mediated interactions with laminin 5.

Reactive oxygen species (ROS) are generally seen as harmful to biomolecules and organisms (Harman, 1956). However, besides the negative effects, ROS are also actively produced and required for many physiological functions in cells (Stone and Yang, 2006; Voeikov, 2006). In addition to the role of ROS in pathogen killing and angiogenesis (Segal, 2005; Ushio-Fukai, 2006), the production of ROS has also been reported to be important in cellular stress conditions. Stimuli that induce the production of free radicals are known to have anti- or pro-apoptotic effects, depending on the duration of the stress (Liu et al., 2005; Reinehr et al., 2006;

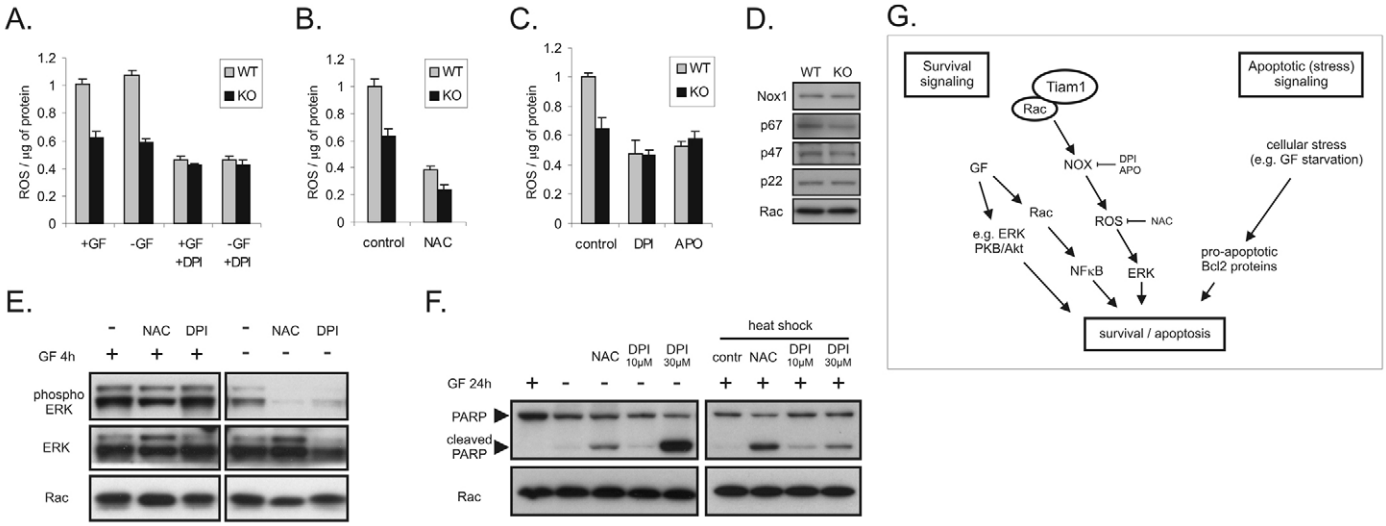

Fig. 6. Nox-dependent ROS production controls ERK phosphorylation and survival. (A) The levels of intracellular ROS were quantified by DFC fluorescence measurement in cell lysates of WT and Tiam1-KO keratinocytes. WT and Tiam1-KO keratinocytes were cultured in the presence or absence of GFs for 4 hours and treated with the Nox inhibitor DPI (15 mM) in the last 30 minutes. Measurements were conducted as described in Fig. 4B. (B) Quantification of intracellular ROS levels in WT and Tiam1-KO keratinocytes treated with or without the antioxidant NAC (10 mM). NAC treatment was carried out for 30 minutes prior to analysis. (C) Quantification of intracellular ROS levels in WT and Tiam1-KO keratinocytes treated with or without the Nox inhibitor DPI (15 mM) or Apocynin (APO, 10 mg/ml) in the last 30 minutes. (D) Western blot showing expression of various Nox components (Nox1-Mox1, p67-phox, p47-phox and p22-phox) in WT and Tiam1-KO cells, Rac expression serves as loading control. (E) Immunoblot showing ERK phosphorylation in WT keratinocytes in control or in GF-deprived conditions (4 hours), in the presence or absence of NAC (10 mM) or DPI (15 µM). Total ERK and Rac expression were used as loading controls. The data are a representative example of two independently performed experiments. (F) Western blot showing PARP cleavage in WT keratinocytes GF-starved for 24 hours or heat shocked (90 minutes at 43°C). Cells were treated with NAC or DPI at the start of the experiment. Rac expression serves as a loading control. The figure is a representative example of four independently performed experiments. (G) Model of the regulation of cellular fate by survival and pro-apoptotic signaling. An apoptotic stimulus (e.g. GF starvation) induces the activation of Bcl2 pro-apoptotic proteins. Tiam1 acts in the survival pathway by controlling Rac-dependent Nox-mediated ROS production and ERK activation. GFs may provide survival signals independent of Tiam1-Rac signaling, leading to ERK and/or Akt or NFκB activation.

Papaiahgari et al., 2006). One of the ROS downstream signaling targets is ERK. ROS-mediated ERK activation has either pro-survival or pro-apoptotic effects depending on the concentration of oxidative radicals and the cell types tested (Guyton et al., 1996; Wang et al., 1998; Chamulitrat et al., 2003; Zhuang and Schnellmann, 2006). However, in most instances ERK activation has a pro-survival function (Henson and Gibson, 2006). We found that ROS-mediated ERK activation in Tiam1-deficient keratinocytes is necessary to prevent apoptosis induced by GF starvation. Interestingly, Tiam1/Rac-mediated ERK activation and survival signaling is not required in normal growth conditions when various GFs are providing survival signals. Tiam1/Rac-mediated survival signaling, however, becomes apparent in stress situations, e.g. upon GF starvation.

The function of Rac proteins in the regulation of Nox enzymes and ROS production has been described previously. Hematopoietic cells like neutrophils and macrophages use Rac1 and especially Rac2 (the hematopoietic-specific Rac isoform) for the activation of gp91^{phox} (Nox2) and the delivery of the oxidative burst (Minakami and Sumimotoa, 2006). In non-hematopoietic cell types, both Rac1 and Rac3 may control ROS production, as demonstrated in primary mouse embryonic fibroblasts (Dolado et al., 2007). Rac1 binds the Nox activator Noxa1/p67^{phox}, and activated GTP-loaded Rac1 is necessary for Nox1 and Nox3 activation (Cheng et al., 2006; Ueyama et al., 2006). However, little is known about the activators of Rac that function in Rac-mediated Nox activation. Here, we show that Tiam1 is involved in Rac1/Nox-dependent ROS production and ERK-mediated survival signaling in keratinocytes independent of normal growth factor signaling. In human neutrophils, Rac2 activation and Nox2 complex formation leading to ROS production was shown to correlate with phosphorylation of Vav1, suggesting a function of Vav1 in ROS production in neutrophils (Zhao et al., 2003). In Caco-2 and HEK293T cells, growth factor-induced ROS production (PDGF and EGF) was dependent on βPix-mediated Rac-Nox activity (Park et al., 2004), whereas Vav2 was shown to be involved in Nox-dependent ROS production in kidney mesangial cells (Chen et al., 2007). Apparently, specific Rac-GEFs can contribute to the activation of Nox and, thereby, ROS production. In COS-phox cells that overexpress all components of the Nox2 complex, a hematopoietic-specific GEF, Vav1, was more efficient in inducing ROS production than other GEFs, such as Vav2 or Tiam1 (Price et al., 2002).

The prevention of apoptosis is a necessary step in tumor initiation. Tiam1-deficient mice are resistant to the initiation of skin tumors induced by oncogenic Ras and intestinal tumors induced by the canonical Wnt signaling pathway (Malliri et al., 2002; Malliri et al., 2006). Previously, we found an inverse correlation between Tiam1-dosage and apoptosis in the epidermal keratinocytes of mice treated with DMBA (Malliri et al., 2002). These findings are consistent with the present data that Tiam1 functions in survival signaling mediated by Nox-controlled ROS production in a dosage-dependent manner. Oncogenic Ras requires Rac for efficient cell transformation (Qiu et al., 1995), and induces ROS production by a Rac-dependent mechanism (Irani et al., 1997). Tiam1 can bind activated Ras directly (Lambert et al., 2002) and thereby may regulate the Ras-mediated Rac activation required for ROS-controlled survival signaling. Thus, Tiam1 may act in the survival signaling pathways that prevent apoptosis in response to both oncogenic and stress signals.

Materials and Methods

Cell culture

Keratinocytes were isolated from the skin of newborn FVB mice and immortalized by SV40 large T antigen expression (Mertens et al., 2005). Cells were cultured in plastic dishes coated with collagen IV (Becton Dickinson) in EpiLife keratinocyte medium (Cascade Biologics) supplemented with 20 μM $CaCl_2$ and EpiLife Defined Growth Supplement, which consisted of EGF, insulin-like growth factor, hydrocortisone, prostaglandin and transferrin (Cascade Biologics). For the suspension assay, cells (5×10^5) were trypsinized and incubated in ultra-low cluster (ULC) six-well cell culture dishes (Costar). Where indicated, cells were treated with MEK inhibitor-PD98059 (Calbiochem), antioxidant N-Acetyl-Cysteine (NAC) (Sigma-Aldrich) or the NADPH oxidase inhibitors: diphenyleneiodonium chloride (DPI) (Calbiochem) and Apocynin (Calbiochem).

Expression vectors, cell transfection and retroviral transduction

Keratinocytes were transfected, using FuGENE (Roche), with pSuper vectors containing short hairpin RNA (shRNA) targeting Tiam1, or luciferase as a control (Malliri et al., 2004). Full-length Tiam1 and catalytically inactive Tiam1-ΔDH coding sequences (Michiels et al., 1997) were cloned into the LZRS-IRES-blasticidin retroviral vector (Michiels et al., 2000). Retroviral constructs were transfected into Phoenix ecotropic packaging cells, and retrovirus-containing supernatants were collected and used for infections, as previously described (Michiels et al., 2000).

Apoptosis assays

Apoptosis was induced in cell cultures at 60-80% confluency. Growth factor (GF) starvation was for 24 hours or as otherwise indicated in the legends to the figures. In heat-shock experiments, cells were incubated for 90 minutes at 43°C, then cultured for 6 hours at 37°C. Subsequently, cells were harvested for the quantification of apoptosis.

For DNA profile analysis, cells were harvested by trypsinization, fixed with cold 70% ethanol, washed with PBS and stained with propidium iodide (PI); flow cytometric analysis was then performed using a FACScallibur (Becton Dickinson). For annexin-V/PI staining, cells were harvested by trypsinization, washed twice with cold PBS, and resuspended in annexin-binding buffer (10 mM Hepes/NaHO, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a final concentration of 1×10^6 to 1×10^7 cells/ml. Subsequently, 200 μl aliquots were stained with annexin-V-APC (Becton Dickinson) and PI, and analyzed using a FACScallibur (Becton Dickinson).

Free nucleosome quantification was performed using the Cell Death Detection ELISA Kit (Roche) according to manufacturer's instructions. For the measurement of PARP cleavage, floating cells were collected by centrifugation and lysed together with the adherent cells in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). Lysates were used for protein content determination, using the BCA protein assay kit (Pierce), and for western blotting with PARP-specific antibody.

Rac activity assay

Rac activity was determined as described previously (Malliri et al., 2004). GTP-bound, active Rac (Rac-GTP) was precipitated from cell lysates by using a biotinylated Rac1-binding domain of PAK1. Total Rac was used as a control.

ROS visualization and quantification

The amount of intracellular reactive oxygen species (ROS) was visualized by dichlorofluorescein diacetate (DFC) (Calbiochem). The cleavage product of DCF, 2′,7′-dichlorofluorescein, is fluorescent upon oxidation by reactive oxygen species (Rosenkranz et al., 1992). Briefly, cells were cultured in 12-well plates. The culture medium was removed and replaced with PBS supplemented with 10 μM DCF for 10 minutes. Subsequently, cells were washed with PBS and images acquired from three or more randomly chosen fields using an epifluorescence microscope (Zeiss, Axiovert 25) equipped with a digital camera AxioCam MRc (Zeiss) and the software MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH). Quantification of the total amount of intracellular ROS was performed in a similar way. Adherent cells were washed with PBS and loaded with DCF 10 μM for 10 minutes. After washing, the cells were lysed in buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 300 mM sucrose, 1% Triton X-100, pH 7.4) and the fluorescence of the lysates determined in a Wallac 1420 Victor2 multipliable reader (PerkinElmer) with an excitation wavelength of 490 nm and an emission wavelength of 530 nm. The readouts were normalized for total protein content, as determined with the BCA Protein Assay Kit (Pierce), and the ROS levels of WT cells was set at 1. When the amount of ROS per cell was determined by FACS analysis, cells were first trypsinized and then loaded with DCF in PBS for 10 minutes, washed, and analyzed using a FACScallibur (Becton Dickinson) in FL1 channel.

Western blotting and antibodies

For western blotting, cells were washed with cold PBS and lysed in RIPA buffer supplemented with 1 mM NaF, 2 mM Na_3VO_4 and a protease inhibitor cocktail (Sigma). The BCA Protein Assay Kit (Pierce) was used to determine protein content. Equal amounts of protein were resuspended in 1×NuPAGE LDS sample buffer (Invitrogen) and separated on NuPage 4-12% polyacrylamide gels (Invitrogen). Proteins were transferred to PVDF membranes and stained with primary antibody. Subsequently, proteins were visualized by staining with an appropriate anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences), and by using an enhanced chemiluminescence kit (Pierce). To remove bound antibodies, membranes were stripped by incubation in a low-pH glycine solution (0.2 M glycine, 1% SDS, pH 2.0) for 15 minutes. Immunoblots were performed with the following primary antibodies: Tiam1 (C16, Santa Cruz), Nox1 (Mox1, H-75; Santa Cruz), p47-phox (H-195, Santa Cruz), p22-phox (MW-1843, Sanquin), β-actin (AC-15, Sigma), phospho-IκB ($pSpS^{32/34}$, Biosource), E-cadherin (clone-36, BD Transduction Laboratories), Rac1 (23A8, BD Transduction Laboratories), ERK2 (clone-33, BD Transduction Laboratories), phospho-ERK1/2 p44/42 MAP Kinase (Thr202/Tyr204, Cell Signaling), total ERK1/2 (137F5, Cell Signaling), phospho-Akt (Thr308, Cell Signaling), total Akt and PARP (Cell Signaling). Picture densitometry quantification was performed with Image J software (National Institutes of Health).

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