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Gab1 and SHP-2 promote Ras/MAPK regulation of epidermal growth and differentiation

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In epidermis, Ras can influence proliferation and differentiation; however, regulators of epidermal Ras function are not fully characterized, and Ras effects on growth and differentiation are controversial. EGF induced Ras activation in epidermal cells along with phosphorylation of the multisubstrate docking protein Gab1 and its binding to SHP-2. Expression of mutant Gab1^{Y627F} deficient in SHP-2 binding or dominant-negative SHP-2^{C459S} reduced basal levels of active Ras and downstream MAPK proteins and initiated differentiation. Differentiation triggered by both Gab1^{Y627F} and SHP-2^{C459S} could be blocked by coexpression of active Ras, consistent with Gab1 and SHP-2 action upstream of Ras in this process. To study the role of Gab1 and SHP-2 in tissue, we generated human epidermis overexpressing active Gab1 and SHP-2. Both proteins stimulated proliferation. In contrast, Gab1^{Y627F} and SHP-2^{C459S} inhibited epidermal proliferation and enhanced differentiation. Consistent with a role for Gab1 and SHP-2 in sustaining epidermal Ras/MAPK activity, Gab1$^{-/-}$ murine epidermis displayed lower levels of active Ras and MAPK with postnatal Gab1$^{-/-}$ epidermis, demonstrating the hypoplasia and enhanced differentiation seen previously with transgenic epidermal Ras blockade. These data provide support for a Ras role in promoting epidermal proliferation and opposing differentiation and indicate that Gab1 and SHP-2 promote the undifferentiated epidermal cell state by facilitating Ras/MAPK signaling.

Introduction

Self-renewing stratified epithelial tissues, such as epidermis, maintain homeostasis by balancing cellular proliferation with postmitotic terminal differentiation. Although the factors controlling epidermal growth and differentiation are not fully understood, recent data supports a role for Ras GTPases in this process. Constitutive expression of active Ras and its effector Raf can trigger growth arrest and differentiation in cultured keratinocytes (Lin and Lowe, 2001; Roper et al., 2001). However, other prior in vitro studies report that Ras pathway signaling supports cell proliferation and opposes differentiation (Mainiero et al., 1997; Zhu et al., 1999).

Recent data suggests that these discrepancies could be due to differences in Ras signal strength in cultured cells (Dajee et al., 2002). In tissue, recent work suggests that Ras may act in a spatially localized fashion within basal layer cells to promote proliferative capacity and oppose differentiation (Dajee et al., 2002). Targeting active Ras mutants to epidermis of transgenic mice generates hyperplastic, undifferentiated epidermis (Bailleul et al., 1990; Greenhalgh et al., 1993; Brown et al., 1998; Dajee et al., 2002). Redundancy and embryonic lethality have hindered generation of tissue deficient in all three H, N, and K-Ras isoforms (Umanoff et al., 1995; Johnson et al., 1997; Ise et al., 2000; Esteban et al., 2001). However, expression of a dominant-negative Ras mutant that lowers levels of active Ras/MAPK in epidermis leads to premature differentiation and proliferative failure (Dajee et al., 2002). Together, these data suggest a tentative model in which Ras acts within epidermis to support proliferative capacity and oppose terminal differentiation. Compelling additional support for this model requires alterations in nonredundant components acting either upstream or downstream of Ras in epidermis.

The relative importance of signaling elements controlling Ras function and mediating its effects can vary depending on the cell type (Shields et al., 2000). Receptors for ligands that include growth factors and matrix proteins can activate Ras in many settings through membrane proximal proteins that include Shc, Grb2, and the guanine nucleotide exchange factor Sos (Schlessinger, 2000; Shields et al., 2000). In epidermis, examples of such receptors implicated in Ras

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induction include certain growth factor receptor tyrosine kinases and integrins (Mainiero et al., 1997; Zhu et al., 1999; Sibilia et al., 2000). Of interest, mice with targeted deletion of several receptors capable of activating Ras display the reduced epidermal proliferation seen with transgenic Ras blockade, suggesting their potential involvement in epidermal Ras signaling. Examples of these receptors include the EGF receptor (EGFR)* (Sibilia and Wagner, 1995; Threadgill et al., 1995) and β1 integrin (Brakebusch et al., 2000). Major effectors that initiate signaling cascades downstream of Ras include Raf family members, phosphoinositide 3-kinases (PI3Ks), and RalGDS proteins (Shields et al., 2000). However, the involvement of specific regulatory and effector molecules important in Ras function, depends on cellular setting and tissue type, and the factors important in epidermal Ras signaling are not clearly defined.

Among proteins acting upstream of Ras are the multisubstrate docking protein Gab1 and the SHP-2 tyrosine phosphatase. Gab1 is a member of a docking protein family (Hibi and Hirano, 2000; Guy et al., 2002; Liu and Rohrschneider, 2002) that recruits multiple signaling proteins after binding to and phosphorylation by selected receptor tyrosine kinases (Holgado-Madruga et al., 1996; Weidner et al., 1996; Lock et al., 2000). Less well characterized as an upstream regulator of Ras function than the Shc/Grb2 proteins, Gab1 appears to play a role in activation of Ras effectors, including the MAPK and PI3K signaling cascades. Although growth factor–driven MAPK activation can proceed normally in $Shc^{-/-}$ cells (Lai and Pawson, 2000), it is impaired in $Gab1^{-/-}$ cells (Itoh et al., 2000; Sachs et al., 2000). Gab1 induction of the MAPK pathway in response to growth factors such as EGF is dependent on binding to the SHP-2 protein tyrosine phosphatase (Cunnick et al., 2001). SHP-2 contains two src homology (SH)2 domains at its NH_2 terminus and is encoded by the *PTPN11* gene recently shown mutated in Noonan syndrome, a disease characterized by facial dysmorphology, growth retardation, and cardiac defects (Feng, 1999; Tartaglia et al., 2001). Gab1 activates SHP-2 by targeting it to the membrane in a process dependent on the NH_2-terminal pleckstrin homology (PH) domain of Gab1 (Cunnick et al., 2002). Although SHP-2 can dephosphorylate Gab1, full characterization of the substrates important for SHP-2 function has not yet been accomplished (Yu et al., 2002). Gab1 and SHP-2 knock-out mice die during embryogenesis, hindering the study of adult tissues null for these proteins (Saxton et al., 1997; Itoh et al., 2000; Sachs et al., 2000). However, analysis of in utero and chimeric tissue suggests that Gab1 and SHP-2 play a role in the morphogenesis of epithelial tissues (Qu et al., 1999; Itoh et al., 2000). The degree to which these proteins influence epidermal growth and differentiation through proteins such as Ras is currently unknown.

Here, we provide evidence supporting a role for Gab1 and SHP-2 in promoting Ras/MAPK signaling to enhance epidermal cell proliferation and oppose differentiation. In epidermal cells, overexpression of wild-type Gab1 and SHP-2 extends the duration of MAPK activation in response to EGF. In contrast, dominant-negative Gab1 and SHP-2 mutants reduce endogenous basal levels of active Ras and MAPK and induce differentiation, a process that can be reversed by coexpression of active Ras. In vivo, disruption of Gab1 function in $Gab1^{-/-}$ postnatal epidermis obtained by embryo grafting and in tissue expressing dominant-negative Gab1 and SHP-2 leads to decreased proliferation and enhanced differentiation. Consistent with this,

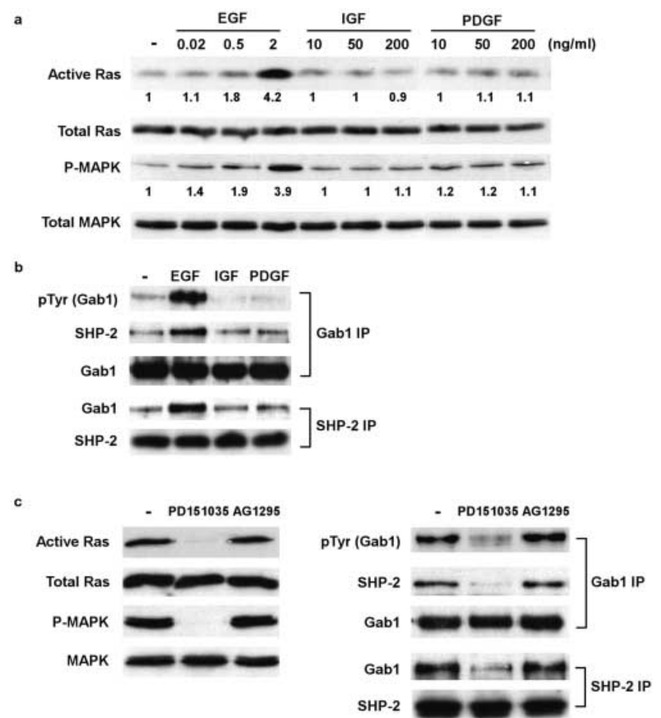

Figure 1. **Selective induction of Gab1 tyrosine phosphorylation and binding to SHP-2 in normal epidermal cells by EGF.** (a) EGF selectively activates Ras/MAPK in primary human keratinocytes. Cells starved in minimal medium for 20 h were treated with EGF, IGF, or PDGF at the concentrations shown. The top panel represents a pan-Ras immunoblot of extracts after GST-RafBD pull-down to isolate only GTP-bound active Ras. Densitometric quantitations of the levels of active Ras and MAPK were proteins normalized to total MAPK loading control and are noted below each corresponding band. The bottom three panels represent immunoblots with the pan-Ras, phosphorylated ERK1/ERK2 MAPK, or total ERK1/ERK2 MAPK antibodies noted at the left of each row. (b) EGF selectively enhances Gab1 tyrosine phosphorylation and binding to SHP-2. Extracts of primary epidermal cells treated with EGF, IGF, or PDGF were immunoprecipitated with antibodies to either Gab1 (Gab1 IP) or SHP-2 (SHP-2 IP) and then immunoblotted with antibodies to Gab1, SHP-2, and phosphotyrosine (pTyr). The EGF-enhanced band recognized on pTyr blotting corresponds to Gab1. (c) Pharmacologic blockade of EGFR function diminishes active Ras/MAPK, Gab1 tyrosine phosphorylation, and binding to SHP-2. The EGFR-selective kinase inhibitor PD151035 and the PDGFR-selective inhibitor AG1295 were incubated with normal epidermal cells grown in complete medium. The levels of active Ras were determined by GST-RafBD pull-down followed by immunoblotting with pan-Ras antibodies and immunoblotting with antibodies to total Ras along with total and active MAPK. Extracts were also immunoprecipitated with antibodies to Gab1 and SHP-2 then immunoblotted with antibodies to Gab1, SHP-2, and pTyr.

*Abbreviations used in this paper: E, embryonic day; EGFR, EGF receptor; IGF, insulin-like growth factor-1; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; SH, src homology.

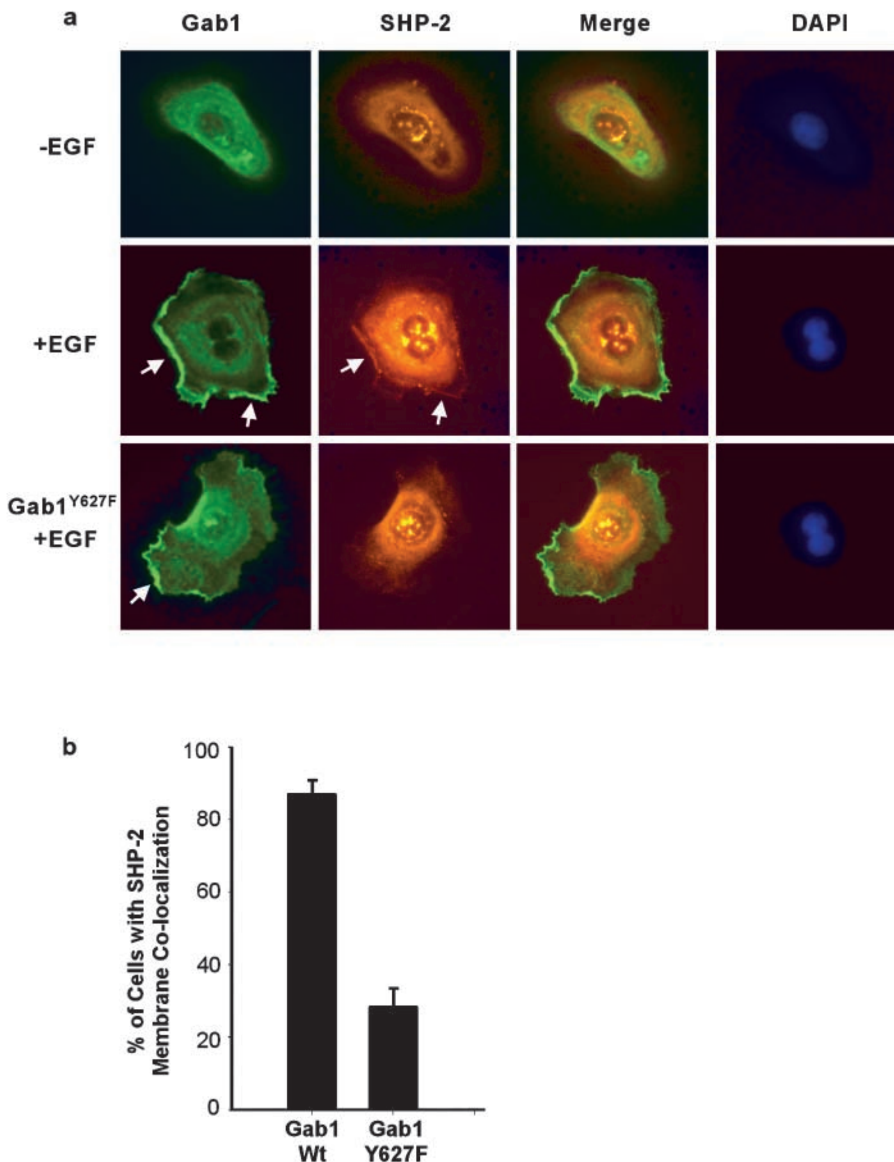

Figure 2. **Interaction between functional Gab1 and SHP-2 in epidermal cells.** (a) Gab1 and SHP-2 localize to the plasma membrane in keratinocytes treated with EGF after 20 h of starvation in minimal medium. Note that Gab1 (green) localizes to the cell perimeter in response to EGF and that SHP-2 (red) fails to do so in the presence of the $Gab1^{Y627F}$ mutant defective in binding to SHP-2. Membrane localization denoted with arrows. (b) Quantitation of the percentage of cells with SHP-2 membrane localization in three independent transductions after EGF treatment.

$Gab1^{-/-}$ epidermis displays diminished levels of active Ras and MAPK. These data indicate that Gab1 and SHP-2 function as nonredundant positive regulators of epidermal Ras function to promote cell proliferation and oppose terminal differentiation.

Results

Selective induction of Gab1 tyrosine phosphorylation and binding to SHP-2 in normal epidermal cells by EGF

Although several growth factor receptors are expressed in stratified epithelial tissues, their relative ability to activate Ras and its effectors such as MAPK in epidermal cells is not fully characterized. To address this, we examined levels of activated GTP-bound Ras and phosphorylated active ERK1/ERK2 MAPKs in primary human keratinocytes treated with three growth factors whose receptors have been implicated in epidermal homeostasis, EGF (Sibilia and Wagner, 1995; Threadgill et al., 1995), insulin-like growth factor-1 (IGF) (Liu et al., 1993), and platelet-derived growth factor (PDGF) (Soriano, 1997). EGF, but not IGF or PDGF, led to a dose-dependent increase in levels of active Ras and MAPK (Fig. 1 a). We next studied the effect of these growth factors on the tyrosine phosphorylation of Gab1 and its binding to SHP-2. EGF, but not IGF or PDGF, led to both increased tyrosine phosphorylation of Gab1 and binding to SHP-2 (Fig. 1 b), suggesting a selective effect of EGF on Ras/MAPK activation mediated by Gab1 and SHP-2. Consistent with this, inhibitors specific for EGFR but not PDGFR lowered basal levels of active Ras and MAPK and decreased both Gab1 tyrosine phosphorylation and Gab1–SHP-2 binding in cells (Fig. 1 c). Thus, in epidermal cells EGF appears capable of activating Gab1, SHP-2, and Ras/MAPK.

Gab1 binding to functional SHP-2 facilitates membrane localization of Gab1–SHP-2 and Ras/MAPK activation

Gab1 binding to functional SHP-2 has been shown to be important for MAPK activation in several cell types (Cunnick et al., 2001, 2002). However, the importance of these

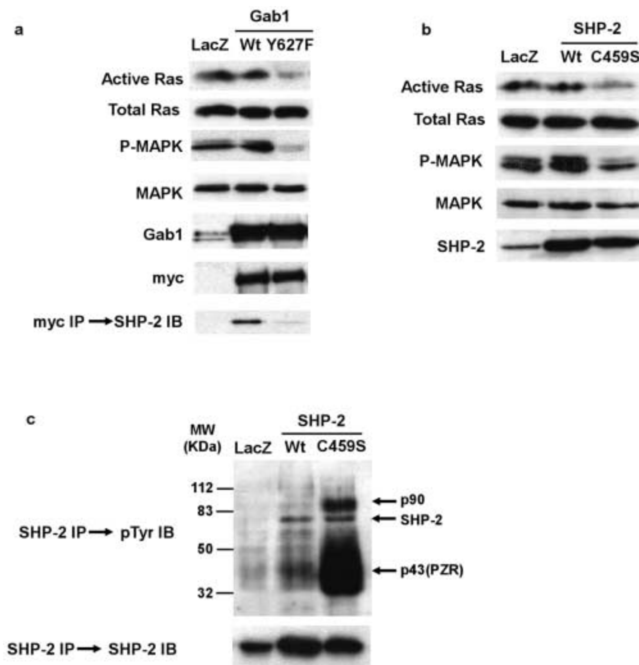

Figure 3. **Effect of Gab1 and SHP-2 on levels of active Ras and MAPK in epidermal cells.** (a) The SHP-2 binding-deficient dominant-negative Gab1^{Y627F} mutant decreases levels of active Ras and MAPK in epidermal cells. Levels of active Ras and MAPK were determined by GST-RafBD pull-down and immunoblotting in primary human keratinocytes transduced at high efficiency with retroviral expression vectors for wild-type human Gab1 and Gab1^{Y627F} along with lacZ transduction control. Cells were grown in complete medium without additional growth factor stimulation. All Gab1 constructs contained a myc epitope tag used to confirm comparable levels of expression and for immunoprecipitation and immunoblotting with antibodies to SHP-2 (myc IP → SHP-2 IB). (b) The dominant-negative SHP-2^{C459S} mutant decreases levels of active Ras and MAPK in epidermal cells. Extracts from primary human keratinocytes transduced with retovectors encoding wild-type SHP-2, SHP-2^{C459S}, and lacZ control were assessed for levels of active Ras, MAPK, and SHP-2. (c) SHP-2^{C459S} binds to increased amounts of tyrosine-phosphorylated proteins in epidermal cells. Extracts from primary human keratinocytes transduced with retovectors encoding wild-type SHP-2, SHP-2^{C459S}, and lacZ control were immunoprecipitated with antibodies to SHP-2. pTyr immunoblotting demonstrates increased association with tyrosine-phosphorylated proteins by phosphatase-defective SHP-2^{C459S}.

processes in facilitating activation of Ras and MAPK in normal epithelial cells has not been studied. In keratinocytes, both Gab1 and SHP-2 localize to the cell perimeter in response to EGF (Fig. 2). SHP-2 redistribution is blocked by expression of the Gab1^{Y627F} mutant defective in SHP-2 binding (Cunnick et al., 2001), indicating that SHP-2 membrane recruitment is dependent on Gab1 in epidermal cells (Fig. 2). Since membrane localization of SHP-2 is sufficient for MAPK activation in other cell types (Cunnick et al., 2002), we examined whether its disruption by expression of Gab1^{Y627F} decreased basal levels of active Ras and MAPK in the absence of growth factor stimulation. Gab1^{Y627F} led to a decrease in constitutively present levels of GTP-bound active Ras and phosphorylated MAPK in keratinocytes (Fig. 3 a). Suppression of basal levels of active Ras/MAPK was also seen with a catalytically inactive dominant-negative SHP-2 mutant, SHP-2^{C459S} (Fig. 3 b). Consistent with prior findings that inactive SHP-2 remains bound to its phosphorylated substrates, immunoprecipitation of SHP-2 in cells expressing SHP-2^{C459S} demonstrated an increase in tyrosine-phosphorylated proteins bound to inactive SHP-2 (Fig. 3 c), including p90 and PZR (Zhao and Zhao, 1998; Shi et al., 2000). Therefore, physical association of Gab1 with a catalytically active SHP-2 facilitates Ras/MAPK activation in epidermal cells.

To examine the duration of MAPK activation as a function of Gab1 and SHP-2 in primary epidermal cells, we determined the kinetics of increased MAPK phosphorylation in response to high dose EGF stimulation in cells transduced with retrovectors encoding Gab1, SHP-2, Gab1^{Y627F}, SHP-2^{C459S}, and LacZ control. Gab1 and SHP-2 overexpression prolonged the persistence of increased MAPK phosphorylation after strong EGF stimulation of cells grown initially in minimal medium without growth factors for 20 h; mutant Gab1 and SHP-2 proteins did not (Fig. 4). Interestingly, dominant-negative Gab1 and SHP-2 fail to block MAPK activation in response to high strength EGF stimulation, suggesting that their capacity for inhibition is confined to lower levels of growth factor action (Fig. 4). These data are consistent with findings in embryonic fibroblasts (Itoh et al., 2000; Shi et al., 2000) that Gab1 and SHP-2 act to sustain MAPK pathway signaling.

Gab1 and SHP-2 affect differentiation gene expression in epidermal cells

If Ras/MAPK regulation is a major effect of Gab1 and SHP-2 proteins, then altering their function should affect epidermal growth and differentiation. These processes can be dramatically influenced by changes in epidermal Ras activity where continual Ras function has been proposed as necessary to prevent differentiation and sustain proliferative capacity (Dajee et al., 2002). Expression of dominant-negative Gab1^{Y627F} and SHP-2^{C459S} but not wild-type proteins induced epidermal differentiation markers in the absence of other stimuli (Fig. 5 a). Differentiation triggered by disruption of Gab1 and SHP-2 function can be blocked by coexpression of active Ras (Fig. 5 b), consistent with a process in which Gab1 and SHP-2 effects on epidermal differentiation reside upstream of Ras. To study Gab1 and SHP-2 effects on differentiation in vivo, we expressed wild-type Gab1 and SHP-2 along with dominant-negative Gab1^{Y627F} and SHP-2^{C459S} in primary human keratinocytes. We then used these cells in a well-established approach (Medalie et al., 1996; Robbins et al., 2001) to regenerate transgenic human skin on immune-deficient mice. Epidermis expressing wild-type Gab1 and SHP-2 displayed slightly decreased differentiation marker staining; however, Gab1^{Y627F} and SHP-2^{C459S}-expressing epidermis displayed markedly enhanced levels of these proteins (Fig. 6 a). These data suggest that Gab1 and SHP-2 influence epidermal differentiation in vivo.

Gab1 and SHP-2 overexpression alters epidermal proliferation in vivo

In addition to opposing differentiation, Ras/MAPK signaling may play an important role in promoting cell prolifera-

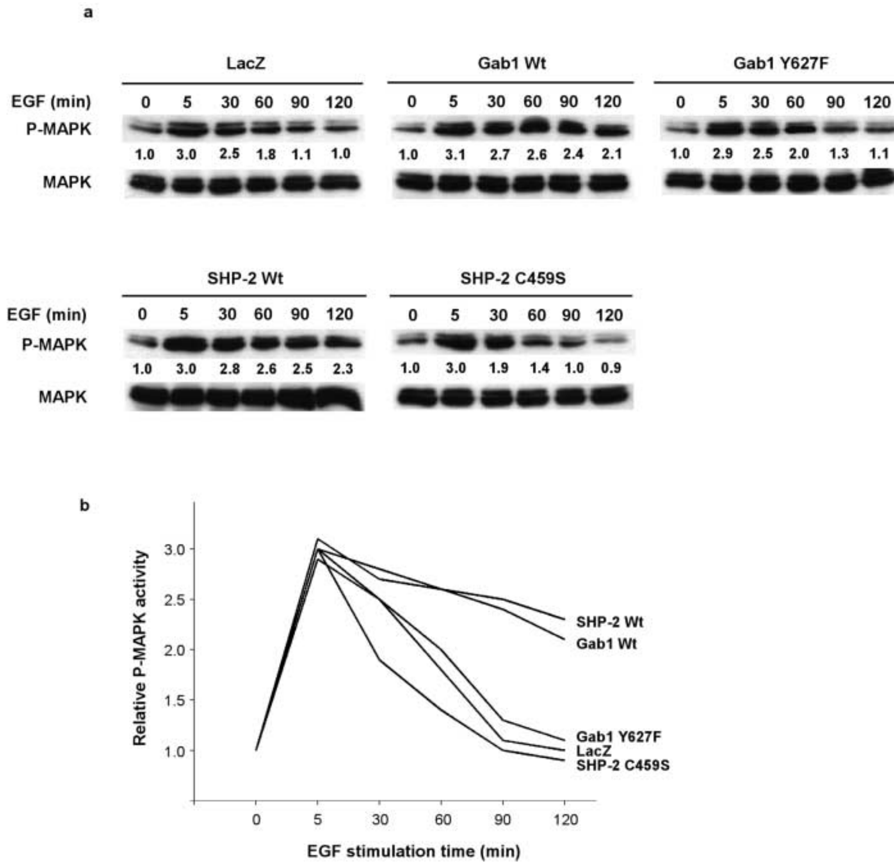

Figure 4. **Gab1 and SHP-2 expression prolongs the duration of elevated active MAPK levels after EGF stimulation.** (a) Levels of total and active MAPK were determined in primary human keratinocytes transduced with retrovectors expressing the proteins noted over each panel. Cells were starved of growth factors for 20 h and then exposed to high dose EGF stimulation at 100 ng/ml for the durations shown. Densitometric quantitation was normalized to total MAPK loading control for each lane and is shown below each corresponding band. Data is representative of three independent time course series of experiments. Wt, wild-type. (b) Graphic representation of active MAPK levels shown in Fig. 4 A over time in cells expressing wild-type and mutant Gab1 and SHP-2 proteins.

tion in epidermis (Mainiero et al., 1997; Zhu et al., 1999; Dajee et al., 2002). To determine if altering Gab1 and SHP-2 function impacts epidermal proliferation, we assessed cell division in transgenic human epidermis. After 4 wk, expression of wild-type Gab1 and SHP-2 led to mild epidermal hyperplasia and an increase in the number of actively dividing cells in vivo. In contrast, Gab1^{Y627F} and SHP-2^{C459S} expression over this period suppressed active cell division and produced epidermal hypoplasia (Fig. 6, a and b). Therefore, Gab1 and SHP-2 can promote epidermal proliferation and dominant interference with their function leads to decreased cell division in vivo.

Altered Ras/MAPK activity and epidermal growth and differentiation in postnatal Gab1$^{-/-}$ tissue

In studying the effects of loss of function of Gab1 and SHP-2, genetic ablation studies are an important complement to expression of transdominant molecules. Shc/Grb2-mediated signal transmission from EGFR to Ras may operate in a partially distinct manner from that mediated by SHP-2 (Shi et al., 2000), and the relative degree to which these two mechanisms function in epidermis is unknown. If Gab1/SHP-2 function is important in maintaining physiologic levels of active Ras, then its disruption should lead to diminished levels of GTP-bound Ras. To test this, we analyzed active Ras levels in epidermis isolated from Gab1$^{-/-}$ embryos. Levels of active Ras and its downstream target MAPKs were decreased in Gab1$^{-/-}$ epidermis compared with Gab1$^{+/+}$ control (Fig. 7 a). Thus, Gab1 plays a nonredundant role in positively modulating Ras/MAPK activity in epidermis.

In agreement with a role for regulation of epidermal Ras function by Gab1 and SHP-2, initial studies of embryonic and chimeric Gab1- and SHP-2–deficient tissue suggest a potential role for both proteins in epithelial morphogenesis (Qu et al., 1999; Itoh et al., 2000). However, Gab1$^{-/-}$ and

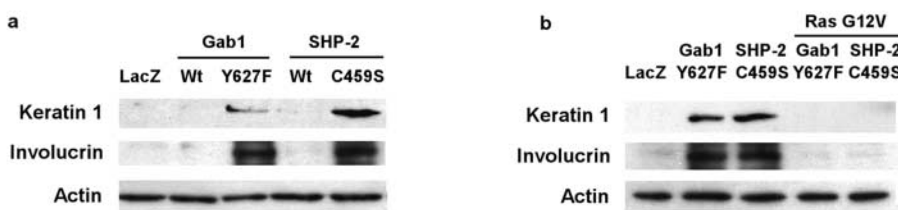

Figure 5. **Effects of altered Gab1 and SHP-2 function on differentiation of epidermal cells.** (a) Expression of dominant-negative Gab1 and SHP-2 induces expression of differentiation markers in primary epidermal cells. Immunoblots of cell extracts prepared from cells expressing wild-type or mutant Gab1 and SHP-2 using antibodies to the differentiation markers keratin 1 and involucrin are shown. Actin loading control for each lane is shown in the bottom panel. (b) Differentiation induced by dominant-negative Gab1 and SHP-2 is blocked by expression of active Ras. Immunoblots of extracts from cells expressing either Gab1^{Y627F} or SHP-2^{C459S} alone or in combination with active Ras^{G12V} are shown.

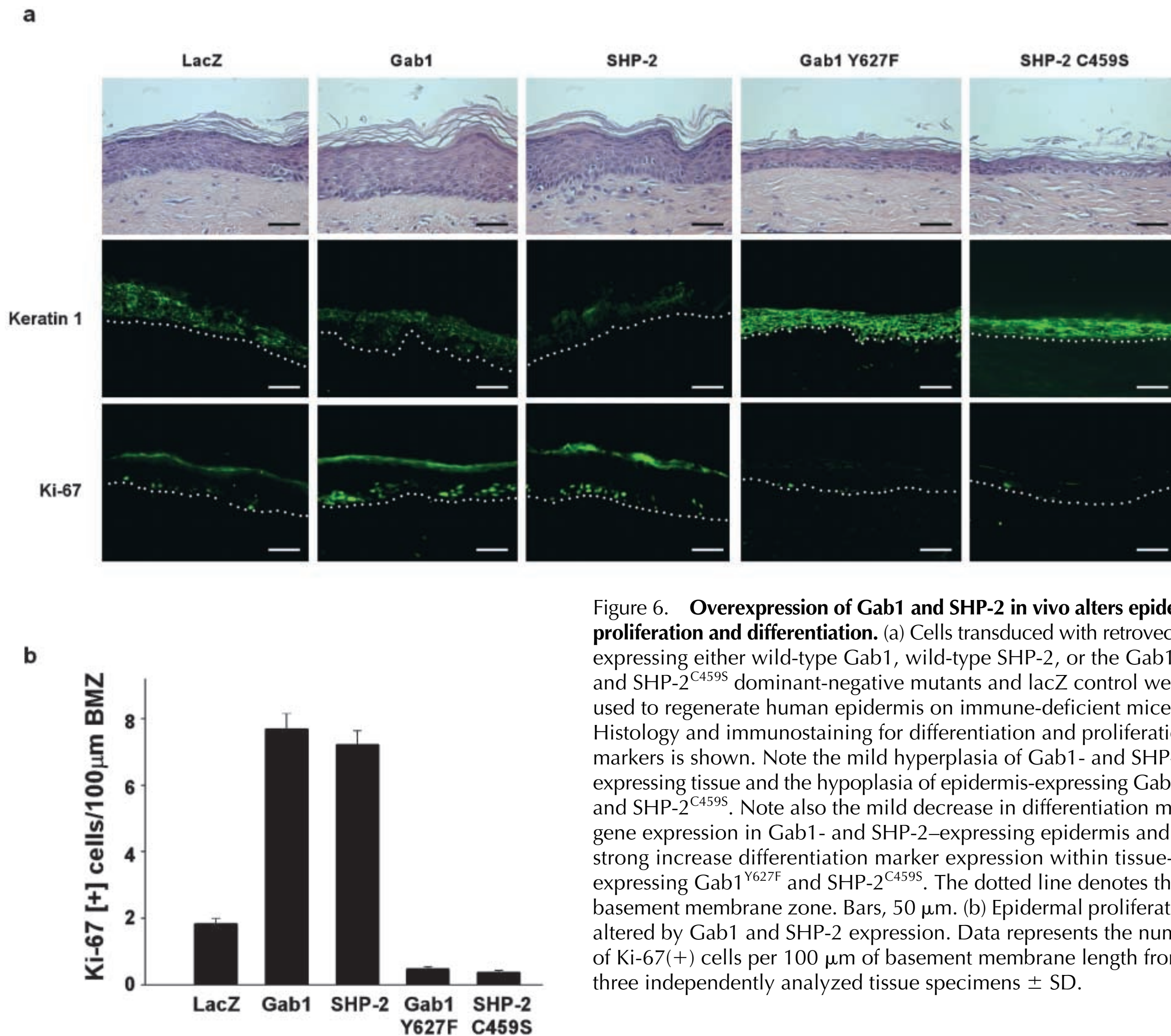

Figure 6. **Overexpression of Gab1 and SHP-2 in vivo alters epidermal proliferation and differentiation.** (a) Cells transduced with retrovectors expressing either wild-type Gab1, wild-type SHP-2, or the Gab1^{Y627F} and SHP-2^{C459S} dominant-negative mutants and lacZ control were used to regenerate human epidermis on immune-deficient mice. Histology and immunostaining for differentiation and proliferation markers is shown. Note the mild hyperplasia of Gab1- and SHP-2–expressing tissue and the hypoplasia of epidermis-expressing Gab1^{Y627F} and SHP-2^{C459S}. Note also the mild decrease in differentiation marker gene expression in Gab1- and SHP-2–expressing epidermis and the strong increase differentiation marker expression within tissue-expressing Gab1^{Y627F} and SHP-2^{C459S}. The dotted line denotes the basement membrane zone. Bars, 50 μm. (b) Epidermal proliferation is altered by Gab1 and SHP-2 expression. Data represents the number of Ki-67(+) cells per 100 μm of basement membrane length from three independently analyzed tissue specimens ± SD.

SHP-2$^{-/-}$ mice die by embryonic day (E)17.5 and E10.5, respectively, hindering phenotypic assessment in postnatal epidermal tissue (Saxton et al., 1997; Itoh et al., 2000). To study the effects of Gab1 deletion in developmentally mature epidermis, we grafted skin from E17.5 Gab1$^{-/-}$ mice along with normal control to immune-deficient mice. 3 wk following grafting after epidermal maturation and the onset of the first anagen hair cycle, Gab1$^{-/-}$ epidermis displayed marked hypoplasia, suggesting a defect in epidermal proliferation (Fig. 7 b). Although basal levels of proliferation are elevated overall in postnatal murine tissue at this time point compared with regenerated human skin, Gab1$^{-/-}$ epidermis displayed a mitotic index of less than half of normal littermate tissue (Fig. 7 c). In addition to proliferative deficits, Gab1$^{-/-}$ epidermis displayed enhanced expression of differentiation markers which in some cases displayed premature expression down to the basal layer (Fig. 7 b), similar to findings seen with epidermis overexpressing dominant-negative SHP-2. Similar findings of lesser magnitude were also observed with E17.5 Gab1$^{-/-}$ skin (Fig. S2 available at http://www.jcb.org/cgi/content/full/jcb.200205017/DC1). The poor growth of Gab1$^{-/-}$ keratinocytes in culture precluded studies to examine the ability of both wild-type and SHP-2 binding-deficient Gab1 to restore Ras/MAPK activation. However, recent work (unpublished data) has demonstrated that introduction of wild-type but not Gab1^{Y627F} into Gab1$^{-/-}$ fibroblast cell lines rescues Ras/MAPK activation. These findings indicate that Gab1 plays a nonredundant role in supporting proliferation and opposing differentiation in the epidermis.

Discussion

Here, we have shown that altering the function of Gab1 and SHP-2 modulates the levels of active Ras/MAPK in epidermal cells in association with changes in growth and differentiation. Differentiation induced by dominant-negative Gab1 and SHP-2 mutants is abolished by active Ras, indicating that Gab1/SHP-2 actions in this process may reside upstream of Ras, consistent with prior biochemical data (Itoh et al., 2000; Shi et al., 2000; Cunnick et al., 2002). Duration of MAPK signaling is important in generating different biologic outcomes, but major cell type differences exist in signaling by Ras and its effectors (Shields et al., 2000). We observed that Gab1 and SHP-2 overexpression prolong the persistence of active epidermal MAPK in

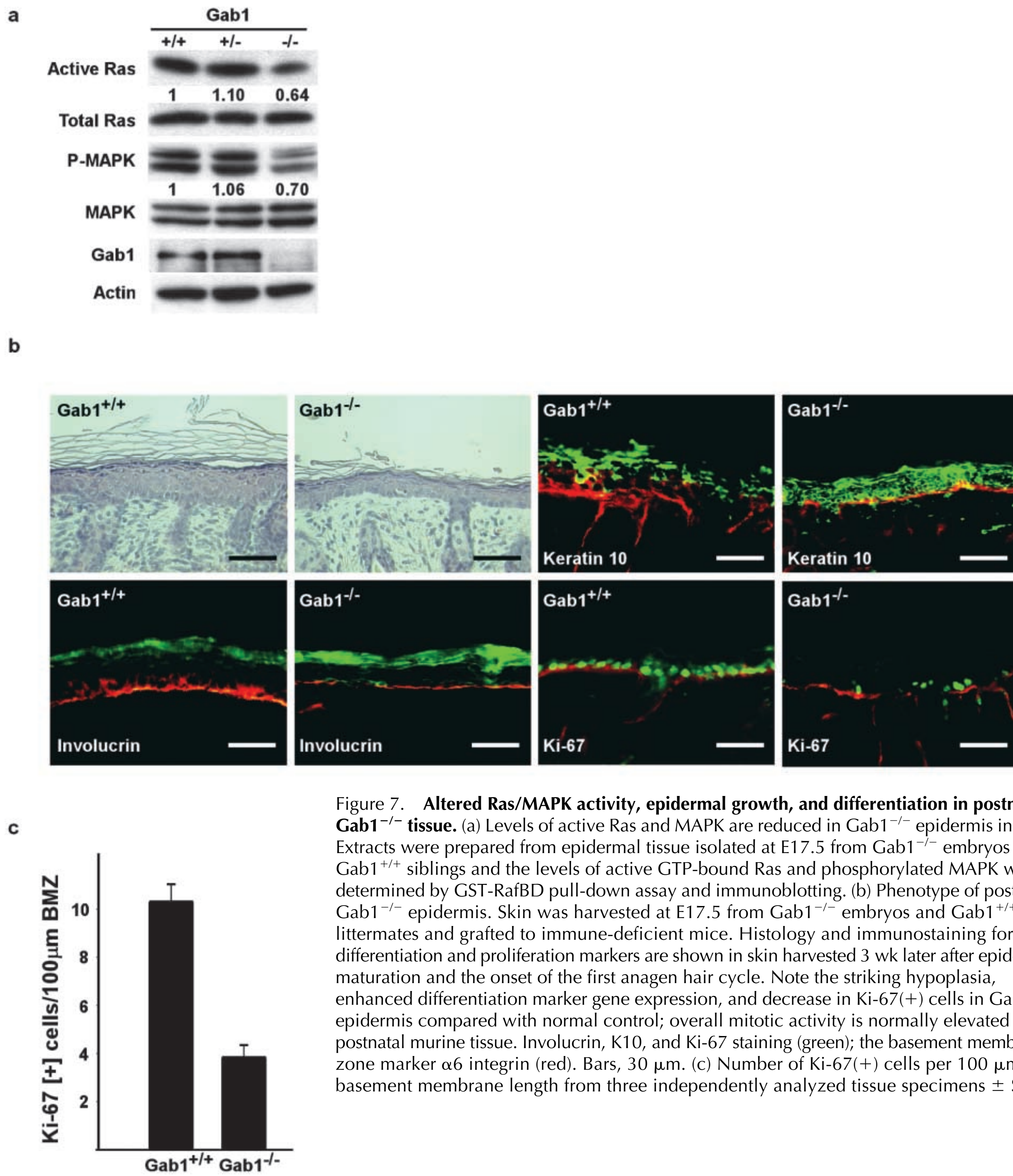

Figure 7. **Altered Ras/MAPK activity, epidermal growth, and differentiation in postnatal Gab1$^{-/-}$ tissue.** (a) Levels of active Ras and MAPK are reduced in Gab1$^{-/-}$ epidermis in vivo. Extracts were prepared from epidermal tissue isolated at E17.5 from Gab1$^{-/-}$ embryos and Gab1$^{+/+}$ siblings and the levels of active GTP-bound Ras and phosphorylated MAPK were determined by GST-RafBD pull-down assay and immunoblotting. (b) Phenotype of postnatal Gab1$^{-/-}$ epidermis. Skin was harvested at E17.5 from Gab1$^{-/-}$ embryos and Gab1$^{+/+}$ littermates and grafted to immune-deficient mice. Histology and immunostaining for differentiation and proliferation markers are shown in skin harvested 3 wk later after epidermal maturation and the onset of the first anagen hair cycle. Note the striking hypoplasia, enhanced differentiation marker gene expression, and decrease in Ki-67(+) cells in Gab1$^{-/-}$ epidermis compared with normal control; overall mitotic activity is normally elevated in postnatal murine tissue. Involucrin, K10, and Ki-67 staining (green); the basement membrane zone marker α6 integrin (red). Bars, 30 μm. (c) Number of Ki-67(+) cells per 100 μm of basement membrane length from three independently analyzed tissue specimens ± SD.

response to EGF stimulation, consonant with prior observations in fibroblasts and transformed cell lines. However, dominant-negative Gab1 and SHP-2 fail to block MAPK induction in response to strong EGF stimulation, underscoring the contribution of other elements such as Sos/Grb2/Shc in this setting.

Combined with induction of differentiation by Gab1/SHP-2 inhibition, these findings suggest that Gab1 and SHP-2 may inhibit differentiation by contributing to sustained Ras/MAPK signaling in epidermal cells. In this regard, the role of Gab1/SHP-2 may be to facilitate basal levels of Ras/MAPK activation in response to factors such as EGF, whereas other proteins, such as Sos/Grb2/Shc, mediate responses to strong growth factor stimulation. In agreement with this possibility is the recent observation that both dominant-negative Ras and pharmacologic inhibition of MEK/MAPK triggers keratinocyte differentiation in the absence of other stimuli (Dajee et al., 2002). These data thus suggest an important role for Gab1 and SHP-2 as necessary in maintaining the undifferentiated state within the epidermis through promotion of Ras/MAPK signaling.

Precise roles for specific receptor tyrosine kinases in controlling growth and differentiation in the epidermis have not been fully defined, and we believe it is unlikely that a

single receptor such as EGFR is the sole control point for these processes. In epidermal cells, we observed that Gab1 tyrosine phosphorylation and binding to SHP-2 along with Ras activation is enhanced in response to EGF and can be blocked by a selective inhibitor of EGFR function. In addition to EGFR, integrins such as β1 appear to promote epidermal proliferation and inhibit differentiation (Brakebusch et al., 2000; Haase et al., 2001), although characterization of integrin effects in isolation from their important roles in adhesion requires further study. Similar to EGFR, specific integrins may rely at least in part on Ras for intracellular signal transmission (Mainiero et al., 1997; Zhu et al., 1999); however, a potential role for Gab1–SHP-2 in integrin signaling has not been systematically examined. SHP-2 does not appear to transmit signal from EGFR to Ras through the better characterized Shc, Grb2, and Sos pathways, but evidence suggests that Gab1 itself may (Shi et al., 2000). Additional studies are required to examine the relative contributions of each of these elements to epidermal growth and differentiation.

Our data suggest that a mechanistic process involving EGFR, Gab1, SHP-2, Ras, Raf, MEK, and MAPK may operate in a manner that is spatially confined to the basal layer of this stratified epithelium. Consistent with this possibility, EGFR, Ras, and active MAPK protein are localized in the undifferentiated basal layer of epidermis (Fukuyama and Shimizu, 1991; Dajee et al., 2002) (Fig. S1 available at http://www.jcb.org/cgi/content/full/jcb.200205017/DC1). Currently, available antibodies to Gab1 and SHP-2 do not function well in tissue immunostaining as judged by failure to give specific signals with skin tissue with either absence or overexpression of these proteins (unpublished data). However, we have shown here that both of these proteins are expressed in undifferentiated epidermal cells, and it has been demonstrated previously that the Gab1 promoter is active in the basal layer of epidermis (Itoh et al., 2000). Since the basal epidermal layer houses the proliferative pool of undifferentiated cells responsible for epidermal self-renewal, such a model of spatially localized action would predict that augmenting the function of components of this pathway in this location would enhance epidermal proliferation. The current work indicates that this prediction holds true for Gab1 and SHP-2. We and others have reported similar effects for Ras (Bailleul et al., 1990; Greenhalgh et al., 1993; Brown et al., 1998; Dajee et al., 2002).

Regarding other components of the Ras/MAPK pathway, epidermal expression of active MEK1 (Haase et al., 2001) also augments proliferation and inhibits differentiation. In further support of our model, interference with function of these proteins leads to diminished proliferation and epidermal hypoplasia. Examples of this include the observations reported here for Gab1 and SHP-2 and previously described $EGFR^{-/-}$ mice (Sibilia and Wagner, 1995; Threadgill et al., 1995) and transgenic mice overexpressing a dominant-negative Ras in epidermis (Dajee et al., 2002). In the case of the latter, hypoproliferation was only observed when dominant-negative Ras was targeted to the basal layer and not suprabasal layer cells, underscoring the spatial localization and basal layer cell intrinsic nature of this process. Together, these data support a model in which epidermal proliferation is influenced by the action of a signaling process involving EGFR, Gab1, SHP-2, Ras, Raf, MEK, and MAPK whose spatial localization helps divide epidermis into a proliferative, undifferentiated compartment and a postmitotic differentiating compartment.

Materials and methods

Cell culture and gene transfer

Coding sequences for human Gab1 (Yart et al., 2001), SHP-2 (Zhao and Zhao, 1998), and $H\text{-}Ras^{G12V}$ were subcloned into the HindIII/NotI, BamHI/HindIII, and BamHI/NotI sites of LZRS vector (Kinsella and Nolan, 1996), respectively. Primary human keratinocytes were isolated and grown as described (Choate et al., 1996). Retrovirus was prepared in human 293T packaging cells as described (Kinsella and Nolan, 1996; Deng et al., 1998). Primary keratinocytes underwent retroviral transduction at a multiplicity of infection of 15 without drug selection (Robbins et al., 2001). Growth factors or receptor inhibitors were incubated with primary human keratinocytes for 5 and 20 min, respectively, before extract preparation at the following concentrations: EGF (10 ng or 100 ng/ml; Sigma-Aldrich), IGF (100 ng/ml; Sigma-Aldrich), PDGF (100 ng/ml; Sigma-Aldrich), PD153035 (250 nM; Calbiochem), and AG1295 (10 μM; Calbiochem).

Protein expression

Keratinocytes were lysed in lysis buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM $MgCl_2$, 1mM EDTA, 10% glycerol) with protease inhibitors and denatured by boiling with ~20 μg of extract loaded per lane. Antibodies were obtained from the following sources: Gab1 and pTyr (Upstate Biotechnology), SHP-2 and c-myc (Santa Cruz Biotechnology, Inc.), pan-Ras (Oncogene Research Products), involucrin and human keratin 1 (Babco), and phospho-MAPK and MAPK (Cell Signaling). Immunoblots were stripped and reprobed with antibodies to β-actin (Santa Cruz Biotechnology, Inc.) as an additional control for loading and extract quality. Results were quantitated using a GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories), and data were normalized to loading control for the same sample lane.

Immunoprecipitation and quantitation of active Ras

500 μg of cell lysate protein was precleared with equilibrated protein A beads (Sigma-Aldrich) and incubated with anti-Gab1 or anti–SHP-2 antibody for 4 h. Immune complexes were precipitated with protein A/G beads (Sigma-Aldrich) and subjected to immunoblotting. The active Ras-GTP pull-down assay was performed under nonsaturated conditions as described (de Rooij and Bos, 1997). Briefly, 150 μl *Escherichia coli* GST-RBD lysate was incubated with 30 μl glutathione-sepharose beads (Amersham Biosciences) at room temperature for 30 min with shaking. After washing the sepharose beads, 500 μg of epidermal cell extract was added at 4°C for 1 h with shaking. After three washes, the samples were subjected to 12% SDS-PAGE. Levels of active Ras protein were detected by a pan-Ras antibody (Oncogene Research Products) and quantitated as noted above.

Histology and immunofluorescence

Genetically engineered human epidermis was regenerated on CB.17 scid/scid mice after gene transfer as described (Choate et al., 1996; Robbins et al., 2001). E17.5 $Gab1^{-/-}$ and wild-type embryonic mice skin was grafted following the same procedure. Four mice were grafted and analyzed per group. At 3–5 wk after grafting, skin tissue was excised and subjected to analysis. For histological examination, skin tissue was fixed in 4% paraformaldehyde overnight, embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. For immunostaining, 5-μm skin cryosections were allowed to air dry for 30 min and permeabilized with cold acetone for 10 min. Sections were blocked with 10% horse serum for 1 h and treated with the primary antibody for 1 h at room temperature. Slides were then washed three times with PBS and incubated for 30 min with secondary antibodies. After three washes with PBS, slides were mounted in Vectashield (Vector Laboratories) and examined under a ZEISS 100M Axiovert microscope. The following panel of antibodies was used in immunostaining: anti–human K1, anti–mouse K10, and anti–mouse involucrin (Babco); anti–human Ki-67 (LabVision); anti–mouse Ki-67 (Dako); anti–integrin α6 (Chemicon); FITC-conjugated goat anti–mouse IgG, FITC-conjugated goat anti–rabbit IgG and FITC-conjugated rabbit anti–rat IgG (Sigma-Aldrich); and Cy3-conjugated goat anti–rat IgG (Jackson ImmunoResearch Laboratories).

Online supplemental material

Figs. S1 and S2 are available at http://www.jcb.org/cgi/content/full/jcb.200205017/DC1. Fig. S1 shows that Ras and EGFR are expressed in the undifferentiated epidermal basal layer. Immunostaining of normal human epidermal tissue with antibodies to Ras and EGFR (green) and the superbasal layer differentiation marker involucrin (red) are shown; the dotted line denotes the basement membrane zone. Fig. S2 shows the histology and immunostaining of proliferation marker Ki-67 and differentiation marker keratin 10 of Gab1 E17.5 embryo skin.

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