The Nuclear Hormone Receptor Peroxisome Proliferator-Activated Receptor β/δ Potentiates Cell Chemotactism, Polarization, and Migration

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After an injury, keratinocytes acquire the plasticity necessary for the reepithelialization of the wound. Here, we identify a novel pathway by which a nuclear hormone receptor, until now better known for its metabolic functions, potentiates cell migration. We show that peroxisome proliferator-activated receptor β/δ (PPARβ/δ) enhances two phosphatidylinositol 3-kinase-dependent pathways, namely, the Akt and the Rho-GTPase pathways. This PPARβ/δ activity amplifies the response of keratinocytes to a chemotactic signal, promotes integrin recycling and remodeling of the actin cytoskeleton, and thereby favors cell migration. Using three-dimensional wound reconstructions, we demonstrate that these defects have a strong impact on in vivo skin healing, since PPARβ/δ−/− mice show an unexpected and rare epithelialization phenotype. Our findings demonstrate that nuclear hormone receptors not only regulate intercellular communication at the organism level but also participate in cell responses to a chemotactic signal. The implications of our findings may be far-reaching, considering that the mechanisms described here are important in many physiological and pathological situations.

Skin repair after an injury proceeds via a pattern of events including inflammation and reepithelialization, as well as matrix and tissue remodeling (43). Reepithelialization requires that the keratinocytes at the wound edge reorganize cell-cell junctions, cell-matrix contacts, and actin cytoskeleton and extend pseudopodia to migrate into the wound. These changes are initiated by signals such as directional sensing of asymmetric extracellular cues, like the members of the epidermal growth factor (EGF) family. Sensing of these directional cues is followed by an internal polarized response, with localized synthesis and accumulation of phosphoinositides, such as phosphatidylinositol-3,4,5-triphosphate (PIP3), at the sites of the membrane receiving the strongest signal (15, 25, 34). The accumulation of PIP3 defines the leading edge, where the polarization machinery is directed. Cell movement then depends on the coordinated assembly and disassembly of actin filaments, which are modulated by the effector pathways of phosphatidylinositol-3-kinase (PI3K) (see Fig. 9) (6, 12, 30, 42). One of these pathways involves Akt1/protein kinase Bε (PKBε), a kinase which contains a pleckstrin homology (PH) domain that binds with high affinity to the PIP3 product of PI3K. Although recent data suggest that its function may depend on the cell type and context (44–46), Akt1 is thought to promote migration by inhibition of glycogen synthase kinase 3β (GSK-3β) (27, 31). A second pathway involves the Rho small GTPase family (17, 29). Among the members of this family of proteins, Rac1 plays a role in the protrusion of lamellipodia and in forward movements, whereas cdc42 maintains cell polarity, including lamellipodium activity at the leading wound edge. Interestingly, Rac1 normal activity is required for efficient wound repair in vivo (40). Furthermore, both Rac1 and cdc42 participate in a positive-feedback loop that increases the PIP3/PIP2 ratio at the leading edge (8, 25), thereby further enhancing localized Akt activity.

Nuclear hormone receptors are ligand-activated transcription factors, which are important regulators of intercellular communication. Upon binding of their specific ligands, they regulate many aspects of the life of multicellular organisms, such as embryogenesis, reproduction, and energy homeostasis, via transcriptional control of gene expression. With the exception of the estrogen receptor (35), the molecular action of nuclear hormone receptors at the level of individual cell behavior such as cell movement is largely unknown. Here we reveal another role of the nuclear hormone receptor peroxisome proliferator-activated receptor β (PPARβ) in cell chemotactic response and migration. We provide evidence that, upon activation by a growth factor, PPARβ−/− keratinocytes are unable to sustain two PI3K/PIP3 effector pathways, namely, the Rho GTPase and the Akt1/GSK-3β pathways (see Fig. 9). In these mutated cells, defects in the activation of Rho GTPases Rac1 and cdc42 and in the inhibition of GSK-3β lead to delayed extension of protrusions towards an extracellular EGF source and to impaired organization of actin stress fibers. In vivo, these defects result in an unusual phenotype of epithelialization of a skin...
wound, with keratinocytes migrating away from the center of the wounded area.

MATERIALS AND METHODS

Reagents. Reagents included anti-kinesin light chain 2 (anti-KLC2; Chemicon); anti-cdc42 (Pierce); anti-keratin 6 (Novocastra); anti-WAVE2, anti-N-WASP, anti-Arp2, anti-phospho-PAK1 (Thr218/223), anti-cdc42, anti-phospho-PAK1 (Ser144)/PAK2 (Ser141), anti-PAK1, anti-PAK2, anti-phospho-LIMK1 (Thr508)/LIMK2 (Thr505), anti-LIMK1, anti-histone H3, and anti-ILK1 (Cell Signaling); antiamphagglutinin (anti-HA) tag (Sigma); rhodamine- phalloidin (Molecular Probes); DAPI (4′,6-diamidino-2-phenylindole) and Vectashield mounting medium (Vector Laboratories); bromodeoxyuridine (BrdU) detection kit and anti-β-III-tubulin tag (Roche); biotinylated goat anti-rabbit antibody (Vector Laboratories); streptavidin-tagged Alexa 488 and Alexa 350 antibodies (Molecular Probes); mouse EGFP (Sigma); porous membrane inserts (BD Biosciences; Falcon); and the Akt kinase assay kit (Cell Signaling). cDNA clones for the dominant-negative and constitutively active mutants of human Rac1 and cdc42 and for the HA-tagged wild-type (wt) Rac1 and cdc42 are from the Guthrie cDNA Resource Center (http://www.ncbi.nlm.nih.gov).

Keratinocyte and skin explant cultures. Primary mouse keratinocytes (passage 3, except for Fig. 1D, passage 0) were cultured and transfected as previously described (37), with dominant-negative or constitutively active Rac1/cdc42, HA-wt Rac1, HA-wt cdc42, or Myc-tagged wt PPARβ/γ, or PH-Akt-green fluorescent protein (GFP) expression vectors. Transfection efficiency ranged between 60 and 80%. At 16 h posttransfection, the cells were replated on a glass-bottomed chamber slide and cultured for 6 h. Directional sensing of the pseudopodia from cell bodies for further analysis was controlled using labeling of the nuclear histone H3. The scraping wound experiments were performed as previously described (26). Fluorescent staining assays were performed as follows. Cells were fixed in 4% formaldehyde–2% sucrose–phosphate-buffered saline (PBS) for 15 min and permeabilized with 0.02% Triton X-100–PBS for 5 min. Blocking was performed by incubating the samples in either 5% normal goat serum-PBS or normal rabbit serum-PBS for 1 h. Actin was stained with rhodamine-phalloidin added at a 1:100 dilution. Nuclei were counterstained with 1:1000 dilution of the entire wounds were stained with mouse anti-keratin 6 at 1/500 and biotinylated goat anti-rabbit secondary antibody at 1/250. Streptavidin-fused fluorescent amplifier Alexa Fluor 488 was added at 1/200, and slides were mounted in Vectashield mounting medium. One out of two sections was imaged using a planapochromatic MZ16 fluorescence stereomicroscope (Leica). Images were imported into Imaris software and its plug-in Autoaligner (Bitplane AG) and aligned manually. Aligned stacks were processed (Imaris) to get the three-dimensional (3D) reconstructed views.

RESULTS

PPARβ/γ keratinocytes show impaired directional sensing and Akt1 signaling in response to EGF. The activation of a cell by a growth factor such as EGF is followed by the activation of the PI3K, by the production of PI3K, at the site of the membrane closest to where the signal is detected, and by the local recruitment and activation of proteins such as Akt via binding of their PH domain to PI3. We used the PI3K domain of Akt, tagged with GFP (PH-Akt-GFP), as a probe to detect the accumulation of PI3 following directional stimulation of PPARβ/γ and PPARβ/γ primary keratinocytes (15, 34).

Upon directional stimulation with EGF supplied locally using a nearby micropipette, transfected PH-Akt-GFP was recruited to a single site of the keratinocyte surface closest to the stimulus, indicating an increase in the PI3/P2P ratio at this site (Fig. 1A). In contrast to the single pseudopod observed in wt cells, PPARβ/γ keratinocytes presented delayed translocation of PH-Akt-GFP and often exhibited several smaller protrusions. Consistent with previous shown reduced phosphorylation of Akt1 (11), in vitro kinase assays showed that the PPARβ-null keratinocytes exhibited lower Akt1 activity than

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FIG. 1. PPARβ-deficient keratinocytes show impaired Rac1/cdc42-mediated translocation of PH-Akt-GFP to the plasma membrane. (A) PH-Akt-GFP-expressing wt or PPARβ−/− keratinocytes were directionally stimulated with EGF. White and blue arrows show recruitment of PH-Akt-GFP to the cell membrane at the leading edge and the retraction of the trailing end, respectively; the asterisks show the point sources of EGF. Bars, 10 μm with EGF. White arrows show recruitment of PH-Akt-GFP to the cell membrane at the leading edge; blue arrows show retraction of the trailing end; the asterisks show the point sources of EGF. 96% of transfected keratinocytes showed delayed recruitment of PH-Akt-GFP to the plasma membrane. Bars, 20 μm. (B) Western blot analysis of active Rac1, cdc42, and Akt1 in wt and PPARβ−/− keratinocytes exposed for the indicated time periods (min) with EGF or PBS alone. Numbers below the Western blots represent the changes in active relative to basal Rac1, cdc42, and Akt1 levels from at least four independent experiments. (C) Western blot analysis of total and phosphorylated PAK1, PAK2, LIMK, GSK-3β, and KLC2 in wt and PPARβ−/− epidermis (left panel; samples from two animals for each genotype are shown) or keratinocytes (right panel) treated for 20 min with either vehicle (V) or EGF (E). Protein levels were normalized to total PAK1, LIMK1, or GSK-3β, and the value 1 was assigned to wt epidermis or vehicle-treated wt keratinocytes. Numbers represent changes (n-fold) relative to corresponding controls from at least four independent experiments. The multiple bands detected for the phospho-PAKs correspond to various phosphorylated forms. p(Thr508)-LIMK1 and p(Thr505)-LIMK2 cannot be distinguished by the antibody and appear as a single band. In panels B and C, the standard deviations of the changes (n-fold) registered were below 7.2% (highest value observed). (D) (Top) PPARβ−/− or PPARβ+/− keratinocytes expressing ectopic Myc-tagged wt PPARβ (PPARβ+/−/Rescue) were transfected with PH-Akt-GFP cDNA and directionally stimulated with EGF. White arrows show recruitment of PH-Akt-GFP to the cell membrane at the leading edge; blue arrows show retraction of the trailing end; the asterisks show the point sources of EGF. Bars, 10 μm. (Bottom) Western blot analysis of active HA-tagged Rac1 and cdc42 in PPARβ−/− keratinocytes and in PPARβ+/− keratinocytes expressing ectopic Myc-tagged wt PPARβ (PPARβ+/−/Rescue), exposed for the indicated time periods (min) to EGF. Numbers below the Western blots represent the changes relative to basal Rac1 and cdc42 levels in the PPARβ−/− cells.

the wt cells did, illustrating a deficient Akt1 pathway in the PPARβ-null cells at the basal level of activity (Fig. 1B, left). Upon stimulation with EGF, the wt cells exhibited a rapid and sustained increase in Akt1 activity compared to a transient and weaker increase in the PPARβ−/− cells (Fig. 1B, right). The defect in cell polarization towards the EGF chemotactic signal was efficiently rescued by transfection of the PPARβ−/− keratinocytes with a wt PPARβ cDNA (Fig. 1D, top). These observations suggest a reduced sensing response to EGF in PPARβ−/− cells, most likely because of an altered internal PIP3 accumulation at the leading edge and, as a consequence, reduced recruitment and activation of Akt1 (Fig. 1A).

GSK-3β is a target of Akt1 known to phosphorylate KLCs and, thus, to negatively regulate kinesin-based motility (27) and integrin recycling (31). Phosphorylation of GSK-3β by Akt1 inhibits its activity, thereby promoting migration in several cell types (14, 16, 19). Concurrently with the reduced Akt1 activity, a lower level of GSK-3β phosphorylation, corresponding to a higher kinase activity, was observed in PPARβ−/− cells (Fig. 1C, see also Fig. 9). As shown in Fig. 1C, KLC2 was hyperphosphorylated in PPARβ−/− cells, which suggests that integrin recycling may be impaired in the PPARβ−/− keratinocytes (see below).

PPARβ−/− cells show reduced activation of the Rho GTPases and of downstream effectors. The small GTPases of the Rho
family are effectors of the PI3K/PIP3 pathway. Among them, Rac1 and cdc42, but not RhoA, were shown to play important roles in directional sensing and migration by driving EGF-induced chemotaxis (17, 29). They activate downstream effectors such as the PAKs, which in turn activate LIM kinases (LIMKs) (21). LIMKs inhibit cofilin, a member of the actin depolymerization factor family (3, 18) (see Fig. 9). Both LIMKs and cofilin are important in processes requiring fast actin reorganization. In the vehicle-treated primary keratinocytes, no difference was seen in the levels of active Rac1 and cdc42 (Fig. 1B, left). wt and PPARβ−/− keratinocytes responded differently to EGF by a sustained and transient activation of two GTPases, Rac1 and cdc42, respectively (Fig. 1B, right). Rescue experiments using ectopic expression of PPARβ in the PPARβ-null keratinocytes efficiently restored the activation of Rac1 and cdc42 (Fig. 1D, bottom). Compared to the PPARβ−/− cells, the PPARβ−/− keratinocytes expressing ectopic PPARβ exhibited a twofold-higher basal Rac1 activity than did the nontransfected cells. When stimulated with EGF, the PPARβ-expressing cells showed a significantly higher increase in Rac1 and cdc42 activity. The level of phosphorylation of PAKs and LIMKs was examined in PPARβ−/− epidermis, as well as in primary keratinocytes treated with either vehicle or EGF. In both cases, PPARβ deficiency led to a reduced phosphorylation of PAK1 (Thr423 and Ser144) and reduced expression and phosphorylation of PAK2 (Ser141 and Ser20) (Fig. 1C). Reduced phosphorylation and hence activity of PAK1 and PAK2 in PPARβ−/− cells were translated into a reduced phosphorylation of LIMK1 and LIMK2 (Fig. 1C). This should impact on the actin cytoskeleton plasticity via cofilin, a hypothesis which will be addressed later in this work.

In summary, the absence of PPARβ causes reduced response to EGF, as exemplified by reduced PI3P accumulation at the membrane and impaired formation of protrusions. Two target pathways of PI3K/PIP3, the Akt1 and the Rho GTPase pathways, as well as their respective downstream effectors GSK-3β and PAKs/LIMKs, are affected in the PPARβ−/− keratinocytes.

Ligand-activated PPARβ potentiates the Rac1/cdc42 pathway. Since the absence of PPARβ reduces Rac1 and cdc42 activities, activation of PPARβ with a selective ligand (L165041) should increase EGF-dependent Rho GTPase activities. Upon exposure to EGF, wt keratinocytes showed a sustained activation of Rac1 and cdc42 (Fig. 2A). The combined treatment with EGF and the PPARβ ligand led to a faster Rac1 and cdc42 activation (Fig. 2A). Consistent with the role of PI3K in the amplification of the internal gradient required to establish cell polarity, this stimulation was neutralized by the PI3K inhibitors LY294002 and wortmannin (Fig. 2B).

In support of these results, coexposure with PPARβ ligand and EGF resulted in a more rapid and robust increase in phosphorylated PAKs (Fig. 2C). Consistent with the reduced expression of PAK2 in PPARβ−/− epidermis and keratinocytes (Fig. 1C), PPARβ ligand treatment increased the expression of total PAK2 in the wt cells (Fig. 2C). Whether this consequence of PPARβ activation for the expression of PAK2 is a direct transcriptional effect was not addressed in the present work. Consequently, the phosphorylation and activation of LIMKs were increased. Thus, the activation of PPARβ by a selective ligand potentiates the cell response to EGF.

Delayed formation of protrusions correlates with decreased actin nucleation and elongation activity in the PPARβ−/− keratinocytes. The above conclusions led us to further analyze cell polarization, the second event in chemotaxis (9). In this, active Rac1 and cdc42 are recruited to the pseudopodium at the leading edge of the cell, where they stimulate PAKs, which in turn leads to the stabilization and growth of the pseudopodium formed de novo. We examined whether PPARβ modulates the formation of this structure using pseudopodium growth through a porous membrane in response to EGF. wt keratinocytes exposed to EGF from below the membrane extended pseudopodia through the pores of the membrane (Fig. 3A). These pseudopodia were detected as early as 30 min after exposure to EGF, and they were still growing 3 hours later. In contrast, in PPARβ−/− cells, the formation of pseudopodia could be detected only after 3 h of incubation (Fig. 3A).

We then quantified the level of active Rho-GTPases in the pseudopodia of PPARβ+/+ and PPARβ−/− cells. In the EGF-stimulated PPARβ+/+ keratinocytes, Rac1 and cdc42 showed increased activity in the pseudopodium fraction, associated with a relative increase in total Rac1 protein, but not in cdc42 (Fig. 3B, left). Pseudopodium extension was severely impaired in wt cells transfected with a dominant-negative mutant of Rac1 (RacT17N) or cdc42 (cdc42T17N) (Fig. 3C). In the PPARβ−/− pseudopodia, the increase in active Rac1 and cdc42 was detected only after a 3-hour incubation, and the induction remained ~2-fold lower than that in wt cells (Fig. 3B). Interestingly, only the overexpression of both constitutively active Rac1 (Rac1G12V) and cdc42 (cdc42G12V) in the PPARβ−/− keratinocytes resulted in significant pseudopodium extension (Fig. 3C). These results revealed that Rac1 and cdc42 fulfill nonredundant complementary functions in pseudopodium growth and that both functions are altered in PPARβ−/− keratinocytes.

Growth of a pseudopodium requires the recruitment of Rac1/cdc42 effectors involved in the nucleation and the elongation of actin filaments such as the Wiskott-Aldrich syndrome protein (N-WASP) and WASP family verprolin-homologous proteins (WAVEs) and the downstream Arp2/3 complex (17) (see Fig. 9). While the amounts of N-WASP and Arp2 proteins were similar in both the cell body and pseudopodium fractions from either wt or PPARβ−/− keratinocytes, communoprecipitation indicated an increase in the effector complex N-WASP/Arp2 in the wt compared with PPARβ−/− pseudopodia (Fig. 3B). Similarly, more WAVE2 was recruited to the extending pseudopodia of PPARβ+/+ than to those of PPARβ−/− cells (Fig. 3B).

These results show that the reduced/delayed activation of Rac1/cdc42 effectors in the PPARβ−/− keratinocytes is associated with reduced N-WASP/Arp2 interaction and WAVE recruitment. These alterations are consistent with the altered actin cytoskeleton dynamic and reduced formation of pseudopodia.

The organization of actin stress fibers and subsequent migration are impaired in PPARβ−/− keratinocytes. Following activation of the cascade of events detailed above, cell movement depends on the coordinated rearrangement of actin filaments (6). All our data converge towards the hypothesis that...
FIG. 2. Ligand-activated PPARβ sustains the activation of Rac1 and cdc42 and of their downstream effectors, PAKs and LIMK. (A and B) Shown are results of Western blot analysis of total and active Rac1 and cdc42 after stimulation with either EGF (0.3 nM), PPARβ ligand L165041 (5 μM), or both (A), with or without EGF in the presence or absence of two PI3K inhibitors, LY294002 (50 μM) and wortmannin (100 μM) (B). Keratinocyte lysates were treated with either excess GDP or nonhydrolyzable GTP-γS, as negative and positive controls, respectively. The ratio of active GTP-bound Rac1 and cdc42 to the total Rac1 and cdc42 protein was quantified in the same protein lysates. Numbers below the Western blots represent the changes in active Rac1 and cdc42 relative to basal active Rac1 and cdc42 from at least six independent experiments. The data shown in panels A and B were obtained in the same experiment; therefore, all the panels can be compared with each other. DMSO, dimethyl sulfoxide. (C) Western blot analysis of total and phosphorylated PAK1, PAK2, and LIMK of wt keratinocyte extracts, treated as described for panel A. Protein expression in vehicle (PBS)-treated cells was normalized to that of β-tubulin and assigned a value of 1. Numbers represent changes (n-fold) in normalized protein expression relative to basal level in vehicle (PBS)-treated cells from six independent experiments. The multiple bands detected for the phospho-PAKs correspond to various phosphorylated forms. p(Thr508)-LIMK1 and p(Thr505)-LIMK2 cannot be distinguished by the antibody and appear as a single band. The standard deviations of the changes (n-fold) given in the panels were below 8.6% (highest value observed).
FIG. 3. Pseudopodium extension by wt and PPARβ−/− keratinocytes. (A) Concentrations of PPARβ+/+ (left panel) and PPARβ−/− (right panel) pseudopodium proteins on the underside of a porous membrane were examined for indicated times in the absence (NT) or presence of EGF in the bottom or top compartment or both compartments. Each point represents the mean ± standard error of the mean of six triplicate membranes obtained from three independent experiments. (B) Western blot analysis of wt (left panel) and PPARβ−/− (right panel) cell body and pseudopodium protein (pseudo P) extracts isolated at indicated times after exposure to EGF in the bottom compartment. Numbers represent the
actin cytoskeleton organization is impaired in PPARβ/−/− keratinocytes. Therefore, the organization of actin filaments and the migration of primary keratinocytes were further studied. In vitro wounds were created by scraping primary keratinocyte monolayers. Striking differences were observed between the PPARβ wt and null keratinocytes in the organization of the cell monolayer and of the actin cytoskeleton. The percentage of keratinocytes that had detached from the wound edge 3 h postscraping in order to colonize the empty space was significantly lower in the PPARβ/−/− than the PPARβ+/+ primary cultures (20.6% versus 43.8% of the edge keratinocytes, respectively). At 3 and 16 h after scraping, the edge formed by the PPARβ/−/− cells remained mostly blunt, with close contacts between cells (Fig. 4, bottom). Moreover, the actin cytoskeleton was cortical and failed to organize into fibers or lamellipodia directed towards the empty space (Fig. 4, top, arrowheads). In contrast, the faster-migrating PPARβ wt keratinocytes lost contacts with neighboring cells, showed well-organized stress fibers, and formed lamellipodia as early as 3 h after scraping (Fig. 4, top, white arrows). Consistently, the in vitro kinetic of healing of the scraping wounds in PPARβ/−/− keratinocyte cultures was delayed compared to that of PPARβ+/+ cultures (70% versus 15% of closure of the empty space by the wt and null keratinocytes, respectively, 16 h after scraping; see also reference 26).

Migration defect of PPARβ/−/− keratinocytes in skin explant cultures. To extend the present study, we turned to an ex vivo model of skin explants that mimics the processes of in vivo wound healing. Skin explants from PPARβ+/+ and PPARβ/−/− newborn pups were grown in culture (24). After 6(430,162),(566,406) days of culture, the efficiency of outgrowth was quantified as the surface covered by keratinocytes migrating out of the explants (Fig. 5A and B). Outgrowth efficiency was dramatically reduced in the PPARβ/−/− compared to that in the PPARβ+/+ samples, with a more-than-threefold smaller surface occupied de novo (wt versus PPARβ/−/−, 35.6 ± 0.8 versus 11.1 ± 0.9 mm²). Consistent with increased proliferation of PPARβ/−/− keratinocytes in vivo (26), their proliferation is also double in the PPARβ/−/− explants (wt versus PPARβ/−/−, 117.4 ± 15.8 versus 216.0 ± 19.4, number of BrdU-positive keratinocytes in the whole outgrowths). We therefore quantified keratinocyte migration only, by measuring the surface covered by keratinocytes in the presence of mitomycin C, which inhibits proliferation. In these conditions too, the outgrowth surface was much smaller in the PPARβ/−/− explants (wt versus PPARβ/−/−, 18.8 ± 2.35 versus 7.6 ± 0.98 mm²), showing that these keratinocytes migrate at least half as efficiently as the wt cells (Fig. 5B).

Morphological analysis of wound biopsy specimens from wt and PPARβ/−/− mice. To analyze the supposed defects in the activation of the sensing signaling cascade and cell migration in vivo, we turned to whole-animal experiments and addressed the impact of PPARβ deficiency on the morphology of the wound edge. At day 4 postinjury, the wt wound biopsy specimens showed a typical early epidermal response, in which the region close to the wound edge exhibited a hyperplastic phenotype that tapered to a two- to three-cell-thick layer at the migratory front (Fig. 6A; see also Fig. S1A in the supplemental material). The wound epidermis was 2.5-fold thicker in the PPARβ/−/− biopsy specimens, with a blunt-ended migratory front (Fig. 6A and B; see also Fig. S1B in the supplemental material). PPARβ/−/− keratinocytes not only migrated less but also receded under the unwounded skin, away from the wound bed (Fig. 6A and D; see also the red arrow in Fig. S1B in the supplemental material). We previously showed that the absence of PPARβ leads to increased proliferation of keratinocytes in mutant mice (26). Since this peculiar directional phenotype may be secondary to keratinocyte increased proliferation and accumulation at the wound edge in the PPARβ/−/− mice, we examined wound edges from Smad3/−/− mice (2), which also showed hyperproliferation but no receding epithelial tongue (see Fig. S1C in the supplemental material). This observation indicated that the receding-tongue phenotype was not the result of the increased proliferation of wound epidermis previously reported (37) but was most likely due to the above-described sensing and migration defects in the PPARβ/−/− cells.

These data indicated that the sensing and migration defects resulting from the absence of PPARβ, described using cell cultures and skin explants, also occur in vivo. Especially striking is the formation of two migratory fronts at the wound edge in the early phase of healing in the PPARβ/−/− animals.

Protein recruitment to the integrin migratory complex is impaired in vivo at the wound edge. Using the same model, we also addressed the consequences of altered signaling in PPARβ/−/− keratinocytes by studying the localization of α3 integrin and of the actin binding protein ILK (integrin-linked kinase). The integrin receptor α3β1 was shown to be an important actor in keratinocyte migration (28). As shown above (Fig. 1C), the phosphorylation levels of GSK-3β and KLC2 suggested that integrin recycling might be impaired in the PPARβ/−/− keratinocytes. Upon activation, α3β1 recruits actin stress fibers through adaptor proteins, among which ILK, a PPARβ direct target product (11), is a major player (see Fig. 9). A similar intensity of the labeling suggested that the expression level of ILK was equivalent in the unwounded epidermis of PPARβ/−/− and PPARβ+/+ animals (Fig. 7A, a’ and b’), whereas it was reduced in the migratory part of the regenerating epidermis 4 days after the wounding of the PPARβ/−/−
compared with the PPARβ+/+ animals (Fig. 7A, a* and b*). The level of expression (data not shown) and the localization (Fig. 7B, Hs) of the α3 integrin subunit were similar in the PPARβ+/+ and PPARβ−/− healthy skin. As expected for PPARβ+/+ skin sections, α3 integrin staining became restricted to the basal plasma membrane of basal keratinocytes located near the initial wound edge and in the migratory layer (Fig. 7B, arrows in panels a* and a*). However, the α3 integrin staining was weaker and diffuse and was seen all around the plasma membrane of the basal PPARβ−/− keratinocytes (Fig.
FIG. 5. Impaired keratinocyte outgrowth from PPARβ−/− skin explants. (A) Representative pictures of keratinocyte outgrowth from PPARβ+/+ (top) and PPARβ−/− (bottom) skin explants after 2 or 6 days of culture, stained by colorimetric development with diaminobenzidine after immunohistochemistry with an anti-keratin 6 antibody. (B) Quantification of the outgrowth surface (mm²) of keratinocytes migrating from PPARβ wt (blue lines) and PPARβ−/− (red lines) skin explants, in the absence (solid line; control) or the presence (dashed line) of mitomycin C to block proliferation.
FIG. 6. Quantification of the thickness, area, and length of the hyperproliferative and migrating wounded epidermis in PPARβ+/+ and PPARβ−/− mice. (A) Keratin 6 staining of the hyperproliferative and migrating epidermis of PPARβ+/+ (top) and PPARβ−/− (bottom) in vivo wounded skin, at day 4 after the injury. The defects in epidermis migration were quantified based on the following parameters: the thickness and the area occupied by the hyperproliferative healing epidermis, the total length of the hyperproliferative epidermis (white triangles), the distance between the initial wound edge and the migratory front (black triangles), and the distance between the initial wound edge and the receding front towards the unwounded tissue (asterisks). HF, hair follicles. (B) Quantification of the thickness of the hyperproliferative epithelium in PPARβ+/+ (blue bar) and PPARβ−/− (brown bar) wounded epidermis, at day 4 after the injury. (C) Quantification of the total area of the hyperproliferative epithelium in PPARβ+/+ (blue bar) and PPARβ−/− (brown bar) wounded epidermis at day 4 after the injury. (D) Quantification of the length of the migrating epithelium in PPARβ+/+ and PPARβ−/− wounded epidermis, at day 4 after the injury. Black triangles, length of the epithelial tongue migrating towards the wound bed; white triangles, length of the total epithelial migrating tongue; asterisks, length of the epithelial tongue receding towards the unwounded tissue. Quantification was performed on three animals of each genotype, on a minimum of five sections per animal. Triangles and asterisks show the values obtained from individual animals; horizontal bars show the average values obtained for each genotype.
FIG. 7. Reduced expression of ILK and impaired localization of α3 integrin in the keratinocytes of a skin wound in PPARβ−/− mice. (A) ILK localization during wound healing. Immunolabeling of ILK was performed on wound biopsy specimens from PPARβ+/+ (a to a′) and PPARβ−/− animals (b to b′) at day 4 after the injury. Neg, negative controls without primary antibody. Higher magnifications are shown in boxes a′ to a″ (PPARβ+/+ samples) and b′ to b″ (PPARβ−/− samples). Black arrowheads indicate the initial wound edge at day 0. Bars, 100 μm (top) and 50 μm (bottom). Immunostaining was performed on three animals of each genotype. HF, hair follicles. (B) α3 integrin localization during wound healing. Immunolabeling of α3 integrin was performed on wound biopsy specimens from PPARβ+/+ (a to a′) and PPARβ−/− animals (b to b′) at day 4 after the injury. Higher magnifications are shown in boxes a′ to a″ (PPARβ+/+ samples) and b′ to b″ (PPARβ−/− samples). Black arrowheads indicate the initial wound edge at day 0. Black arrows in boxes a′, a″, b′, and b″ point to a representative distribution of the α3 integrin subunit. Neg, negative controls without primary antibody; Hs, α3 integrin staining of healthy skin. Immunostaining was performed on three animals of each genotype.

7B, arrows in panels b′ and b″). This is consistent with the impaired localization of the α3 integrin subunit at the leading edge of PPARβ−/− keratinocytes migrating out of skin explant cultures (data not shown).

Altogether, the migratory complex comprising α3β1 integrin and ILK appears to show alterations in the PPARβ−/− animals.

3D reconstruction of PPARβ+/+ and PPARβ−/− in vivo wounds. In order to strengthen the above in vivo observations, we decided to obtain a 3D view of the injured region through imaging and 3D reconstruction. Pictures of serial sections encompassing complete wounds of PPARβ+/+ and PPARβ−/− mice (n = 5) at day 4 after wounding were piled up to reconstruct 3D views of wound edges (Fig. 8). These reconstructions revealed striking differences between PPARβ+/+ and PPARβ−/− wound edges. The PPARβ+/+ epidermal migratory layer was thin as it progressed over the wound bed (Fig. 8a and b; black arrowhead and brown area). No significant receding under the healthy tissue was observed (Fig. 8a and b). In contrast, the PPARβ−/− migratory epidermis showed high variability in its morphology, displaying a receding layer (Fig. 8c and d, asterisk) and thickening of the migratory epidermis (Fig. 8c and d, brown area). These data showed that the impaired migration of the hyperproliferative/migrating epidermis is not only a local random alteration but is also distributed over the entire healing wound edge in the PPARβ−/− mice. Together, these results indicated that the migration defect significantly contrib-
utes to the delayed healing of skin wounds observed in the PPARβ mutant mice.

**DISCUSSION**

The skin is the most important barrier of the body, protecting it from UV or bacterial aggression or from mechanical injuries. Repair of this organ after an injury is thus a life-saving priority. In a skin wound, keratinocytes of the edges are activated by various signals, such as growth factors. The keratinocytes initially detect the asymmetric extracellular cue, which results in the activation of PI3K and accumulation of PIP3 at the cell membrane, a process referred to as directional sensing. The keratinocytes then respond by polarization and extension of protrusions towards the signal. These changes require the activation of signaling cascades that relay sensing to the actin cytoskeleton, whose plasticity is the driving force of cell polarization and migration (6).

We have demonstrated previously that the healing of a skin wound is slower in PPARβ-null mice (26). We further showed that this phenotype is partially due to increased apoptosis of the PPARβ-null keratinocytes (26). Herein we demonstrate the involvement of PPARβ in keratinocyte directional sensing, as well as in the regulation of two PI3K/PIP3 downstream pathways that relay sensing to cell movement, namely, the Rho-GTPase and the Akt pathways. We show that cell directional sensing and activation of these pathways are impaired in PPARβ-/- keratinocytes. Consistently, we demonstrate that the plasticity of actin cytoskeleton is altered in the absence of PPARβ. These defects contribute significantly to affected epithelialization and to delayed healing of skin wounds in the absence of PPARβ in vivo.

The nuclear hormone receptor **PPARβ** is an important modulator of cell directional sensing and movement. Nuclear hormone receptors are usually best characterized by their functions in embryogenesis, body growth, reproduction, and energy homeostasis. So far, only the estrogen receptor has been shown to be involved in the regulation of actin cytoskeleton plasticity and cell movement (35). The findings presented herein provide

![3D reconstruction of PPARβ+/+ and PPARβ−/− wounded skin. (a and b) Two different rotation views of the same 3D reconstruction of the wound edge of a PPARβ+/+ animal at day 4 after the injury. (c and d) Two different rotation views of the same 3D reconstruction of the wound edge of a PPARβ−/− animal at day 4 after the injury. Blue, unwounded epidermis; brown, hyperproliferative/migrating epidermis. Dermis is not shown on the figure. Black arrows, direction of the migration towards the wound bed; asterisk, receding hyperproliferative epidermis. 3D reconstruction was performed on three animals of each genotype.](image_url)
an important new insight into nuclear hormone receptor functions. We show that PPAR\(\beta\), otherwise involved in lipid catabolism, potentiates the establishment of the internal signal required for directional sensing towards a cue such as EGF, as well as the activity of Akt1/GSK-3\(\beta\) and Rac1/cdc42 and their downstream effectors (Fig. 9). PPAR\(\beta\) acts on several steps of the Akt1/GSK-3\(\beta\) and Rac1/cdc42 pathways (Fig. 9). We previously demonstrated that PPAR\(\beta\) decreases the expression of PTEN while directly increasing the expression of PDK1 (11). We also showed that, by downregulating the expression of PTEN, PPAR\(\beta\) potentiates the production of PIP\(3\) (11). Therefore, the coordinated action of PPAR\(\beta\) on PTEN/PIP\(3\) production and PDK1 expression indirectly results in increased Akt1, PAK, and Rac1/cdc42 activity and in increased directional sensing efficiency, as well as enhancement of the EGF signaling (3, 20). Finally, we showed that PPAR\(\beta\) directly activates the expression of ILK (11), thereby potentiating integrin receptor-dependent signaling. These defects have significant consequences for actin cytoskeleton reorganization and integrin recycling (see below), with consequences for cell movement in cell culture and, importantly, also in vivo.

Because impaired PIP\(3\) signaling in PPAR\(\beta\)-null keratinocytes is due to increased expression of PTEN, with consequently reduced PIP\(3\) levels (11), this defect concerns all signals that act on the PI3K pathway upstream of PTEN and, therefore, is not specific to the EGF response. In line with this, we have shown that a similar defect has also been observed in response to insulin-like growth factor (data not shown) and that cultured primary keratinocytes show increased PTEN expression and a reduced amount of PIP\(3\) in the absence of any challenge (11). Therefore, PPAR\(\beta\)-null keratinocytes have impaired basal PI3K signaling, a defect which is exacerbated in challenging situations, such as the response to a chemotactic signal, and further influences cell migration efficiency.

PPAR\(\beta\) modulates cell polarization and migration. The downstream consequence of cell directional sensing at the leading edge of a wound is the recruitment of specialized proteins required for actin filament plasticity and pseudopodium projection. This includes the Rho-GTPase effectors WAVE-WASP-Arp2/3 complexes, involved in de novo actin filament nucleation and elongation, and PAKs/LIMK/cofilin, required for fast actin reorganization (17, 18). PPAR\(\beta\)\(^{-/-}\) keratinocytes showed reduced active Rac1/cdc42, which translated to less-active Arp2/3 and LIMK. We demonstrated that...
the decreased activity of these proteins led to the delayed polarization and formation of pseudopodia in PPARβ−/− primary keratinocytes.

PPARβ also plays a role in the localization of integrin receptors at the cell membrane, which is crucial for cell adhesion and migration and for relaying signals between the extracellular matrix and the actin cytoskeleton (41). As discussed above, the absence of PPARβ results in lower activity of Akt1 and consequently in increased activity of GSK-3β. Consistent with the role of GSK-3β in inhibiting integrin recycling via phosphorylation of KLC (27, 31), the localization of the α3 integrin subunit is impaired in PPARβ−/− cells. Importantly, the PPARβ direct target gene product ILK is part of the complex implicated in integrin interaction with actin (4, 11), and it was recently shown to be required for epidermal morphogenesis, keratinocyte adhesion, and directional migration (23). ILK expression is decreased in PPARβ−/− keratinocytes, which most probably affects integrin-actin interactions. It is interesting that the active Rac1/cdc42 profile observed in PPARβ−/− keratinocytes is similar to that reported for keratinocytes with impaired α6β4 integrin expression (32). Recently, we have shown that PPARβ-stimulated Akt1 signaling resulted in both a profound redistribution of integrins in human proximal tubular epithelial cells (HK-2) and protection against apoptosis during ischemic acute renal failure (22). Labeling of primary or explant keratinocytes also showed that in PPARβ−/− cells, actin tends to locate at the periphery of the cells, suggesting that the actin filaments remain associated with cell-cell junctions rather than being reorganized in order to allow cell movement. Thus, the impaired migration of PPARβ−/− keratinocytes is most likely the consequence of altered actin cytoskeleton dynamics, decreased ILK activity, and disturbed integrin localization and functions.

The absence of PPARβ has severe consequences for the epithelialization stage of skin wound healing. PPARβ modulates keratinocyte directional sensing, as well as migration via actin cytoskeleton plasticity and integrin function. Decreased expression of ILK and impaired localization of the α3 integrin subunit were observed in vivo, which strongly suggests that these pathways are affected in vivo in the absence of PPARβ. The consequence of impaired chemotaxis and migration in vivo is the formation of two migration fronts in skin wounds of PPARβ−/− mice. One of them recedes towards the unwounded tissue, and the other, although correctly oriented towards the wound bed, is twice as short as that in wt animals, with stacking of keratinocytes in a thicker migratory tongue. As in the PPARβ-null mice, impaired epithelialization usually leads to delayed closure of the wound area. However, migration of keratinocytes away from the wound bed is a peculiar phenotype that has been reported only once so far, during the healing process of skin wounds in the fibrinogen-null mouse (13). In the PPARβ-null mice, this phenotype was particularly striking when comparing 3D reconstructions of whole-skin wounds from PPARβ wt and PPARβ−/− animals. Importantly, receding epithelial tongues were not seen in Smad3−/− animals, and the slower migration of PPARβ keratinocytes was still dramatic in skin explants after inhibition of proliferation. This indicates that the phenotype observed in the PPARβ−/− wounds is not primarily due to the increased proliferation of the wound epidermis that was observed previously (37) but to the sensing and migration defect in the PPARβ−/− cells.

Like most of the other nuclear hormone receptors, PPARβ is activated through binding to an agonist. We previously showed that treatment of primary keratinocytes with inflammatory cytokines not only induced the upregulation of PPARβ expression but also stimulated the production of an endogenous ligand, unidentified so far (37). In vivo, the injury-triggered release of inflammatory cytokines reactivated the expression of PPARβ (26) and probably generated the production of the physiological agonists necessary for PPARβ activation in the keratinocytes at the wound edges. Therefore, the activity of PPARβ results from both its increased expression and the production of ligands. Although the nature of such ligands remains to be identified, it is tempting to hypothesize that COX2 derivatives may be involved in PPARβ activation. COX2 expression is known to be increased in inflammatory conditions, and we demonstrated that it generates PPARβ agonists during hair follicle maturation in mouse pups (10). However, this does not rule out the involvement of other pathways, and further work is needed to isolate the physiological agonists that trigger PPARβ activity in the epidermis.

Our results do not exclude the possibility of other confounding defects in keratinocyte migration or in the production of chemotactranscators per se. In addition, the above-mentioned defect in the control of keratinocyte proliferation, the underlying mechanism of which is presently under investigation, certainly participates in the delayed reepithelialization observed in PPARβ−/− animals (26). The main finding of this work is a newly reported function of PPARβ, a member of the nuclear hormone receptor family, in modulating keratinocyte chemotactic response and migration. PPARβ acts via transcriptional up- and downregulation of gene expression (ILK, PDK1, and PTEN) and consequently via the activation of the PI3K/PIP3 pathway and of two of its downstream routes that involve Akt1/GSK-3β, Rac1/cdc42 GTPases, and PAKs. Thus, PPARβ actively participates in the reorganization of the actin cytoskeleton of keratinocytes at a wound edge and is directly involved in the early response of these cells to injury-induced stimuli. The sensing defect due to the absence of PPARβ in vivo causes an unusual epithelialization impairment in the early phase of healing, with the formation of a second migratory front moving away from the wound bed. The role of PPARβ as a regulator of chemotactic response is probably more widespread, since PPARβ is ubiquitously expressed (5), as are the other players of cell migration studied herein. In addition, the question remains open as to whether other nuclear hormone receptors may also fulfill similar functions. Looked at from this viewpoint, the findings presented here may be far-reaching, considering that cell adhesion and movement are fundamental processes involved in a wide range of both physiological and pathological cellular processes, such as morphogenesis in embryonic development, fibrosis, metastasis of tumor cells, and atherosclerosis (1, 38, 39).

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