STAT3 Deficiency in Keratinocytes Leads to Compromised Cell Migration through Hyperphosphorylation of p130cas*

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We previously reported that STAT3 plays a crucial role in transducing a signal for migration of keratinocytes (Sano, S., Itami, S., Takeda, K., Tarutani, M., Yamaguchi, Y., Miura, H., Yoshikawa, K., Akira, S., and Takeda, J. (1999) EMBO J. 18, 4657–4668). To clarify the role of STAT3 in signaling the migration, we studied the intracellular signaling pathway through an integrin receptor in STAT3-deficient keratinocytes. STAT3-deficient keratinocytes demonstrated increased adhesiveness and fast spreading on a collagen matrix. Staining with anti-phosphotyro sine antibody revealed that STAT3-deficient keratinocytes had an increased number of tyrosyl-hyperphosphorylated focal adhesions. Analyses with immunoprecipitation revealed that p130cas was constitutively hyperphosphorylated on tyrosine residues, while other focal adhesion molecules such as focal adhesion kinase and paxillin were not. Transfection of STAT3-deficient keratinocytes with an adenoviral vector encoding the wild-type Stat3 gene reversed not only impaired migration but also the increased tyrosine phosphorylation of p130cas. These results strongly suggest that STAT3 in keratinocytes plays a critical role in turnover of tyrosine phosphorylation of p130cas, modulating cell adhesiveness to the substratum leading to growth factor-dependent cell migration.

Signal transducers and activators of transcription (STATs) are a family of latent cytoplasmic transcription factors that are activated by many cytokines and growth factors (1–3). STATs are phosphorylated on tyrosine residues by activated kinases in receptor complexes, leading to formation of homo- or heterodimers and translocation to the nucleus in which they regulate transcription. STAT3 is activated by a variety of cytokines and growth factors such as interleukin-6, epidermal growth factor (EGF), hepatocyte growth factor (HGF), platelet-derived growth factor, and granulocyte-colony-stimulating factor. These cytokines and growth factors regulate the biological activities of keratinocytes (4, 5), suggesting that STAT3 plays a crucial role in keratinocytes. Because germ line STAT3 deletion leads to embryonic lethality (6), to elucidate the biological roles of STAT3 in the skin, we previously generated keratinocyte-specific STAT3-deficient mice by conditional gene targeting using the Cre-loxP strategy (7). The Stat3 gene was disrupted under the control of a keratin 5 promoter. The mutant mice were born with no apparent abnormalities, and their epidermis and hair follicle development was normal at birth. However, wound healing was markedly retarded, and the second hair cycle was impaired in keratinocyte-specific Stat3 gene knockout mice. An in vitro study with cultured keratinocytes revealed that this phenotype was attributed to impaired migration because of the failure of STAT3 activation.

Cell migration is composed of several concerted steps (8, 9). Migration is initiated with membrane protrusion (filopodia and leading edge) and adhesion to the extracellular matrix, followed by cell traction and the release of adhesions at the rear portion of the cell. These events are regulated by multiple signaling mechanisms, such as tyrosine kinase and/or phosphatase signaling (10–14), mitogen-activated protein kinase signaling (15, 16), small GTPase signaling (i.e. Rho and Rac) (17, 18), and cytoskeletal reorganization (i.e. actin polymerization/dem polymerization, actin/myosin motor) (19, 20). However, it is still undetermined whether STAT3 is involved in these signaling events.

In this report, we found that STAT3-deficient keratinocytes showed increased adhesiveness and forced spreading on a collagen matrix and that an increased number of hyperphosphorylated focal adhesions, in particular, an adaptor protein, p130cas, was constitutively hyperphosphorylated on tyrosine residues. These results strongly suggest that intracellular signaling of STAT3 in keratinocytes modulates tyrosine phosphorylation of p130cas and cell adhesiveness to the substratum, leading to cell migration in response to growth factors.

EXPERIMENTAL PROCEDURES

Generation of Keratinocyte-specific STAT3-disrupted Mice—Keratinocyte-specific STAT3-disrupted mice were generated by using the Cre-loxP strategy under the control of a keratin-5 promoter as previously described (7). Briefly, mice carrying keratin-5 promoter-driven (K5-Cre) transgene and a Stat3-null allele (K5-Cre/Stat3-/-) were mated with Stat3flox/flox mice. Offspring carrying a floxed Stat3 allele and/or K5-Cre transgene (K5-Cre/Stat3flox/+; K5-Cre/Stat3flox/-; Stat3flox/-; Stat3flox/-) were examined for their genotypes by allele-specific PCR. Care of mice was in accordance with institutional guidelines.

Cell Culture—Primary cultures of mouse keratinocytes were established from newborn to 5-day-old mice. The total skin taken from mice was treated with 250 units/ml dispase (Godo Shusei, Tokyo, Japan) overnight at 4 °C, and the epidermis was peeled off from the dermis. Keratinocytes were collected upon trypsinization and washed in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Cells were resuspended in MCDB153 medium containing 3% fetal calf serum and seeded onto type I collagen-coated dishes.

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The abbreviations used are: STAT, signal transducers and activators of transcription; EGF, epidermal growth factor; HGF, hepatocyte growth factor; PBS, phosphate-buffered saline; SH2 and SH3, Src homology 2 and 3, respectively; FAK, focal adhesion kinase; PTP, protein-tyrosine phosphatase.

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RESULTS

STAT3-disrupted Keratinocytes Exhibited an Increased Adhesiveness to the Collagen Matrix—We previously demonstrated that keratinocyte-specific STAT3-disrupted mice showed retardation of skin wound healing and that migration of STAT3-deficient keratinocytes was impaired (7). Cell migration is a coordinated and complex process, including cell adhesion to the extracellular matrix and organization of the actin cytoskeleton. Therefore, we hypothesized that STAT3 is involved in a specific point in these processes. First we compared cell attachment to the extracellular matrix. Freshly isolated keratinocytes were seeded onto type I collagen-coated dishes. Then nonadherent cells were removed by washing at the indicated time points, and attached cells were counted. As shown in Fig. 1a, STAT3-disrupted keratinocytes (white bars) showed significantly increased adhesiveness to the collagen matrix as compared with control cells (black bars) at 20 min, although no difference was observed after 1 or 3 h. The adhesion of keratinocytes to type I collagen is mediated through α5β1 and/or αvβ3 integrins, and the STAT3 signaling pathway may modulate integrin expression (22) in some cells. However, no difference in integrin expression was found between control and STAT3-disrupted keratinocytes (Ref. 7 and data not shown). Therefore, increased adhesion of STAT3-disrupted keratinocytes was not attributed to increased integrin expression.

STAT3-disrupted Keratinocytes Spread Faster on the Collagen Matrix—Next, we compared cell spreading on the substrate of STAT3(−/−) keratinocytes with controls. Twenty min after seeding, an increased number of STAT3(−/−) keratinocytes exhibited coarse and strong signals on the surface (Fig. 1b, black arrows), which is a characteristic feature of spread cells, whereas control keratinocytes (STAT3(+/−)) were mostly round and phase-bright (nonspread) (Fig. 1b, upper panel). Quantitative evaluation revealed that the number of spread cells in STAT3-disrupted keratinocytes was 5-fold that at 20 min compared with controls, although no difference in cell spreading was observed between the two kinds of keratinocytes later than 1 h (Fig. 1c). Taken collectively, these results indicate that STAT3-disrupted cells were primed for attachment and spreading on the substratum, implying that STAT3 plays a critical role in regulating subsequent cell motility through integrin/focal adhesion signaling.

An Increased Number of Tyrosine-phosphorylated Focal Adhesions in STAT3-disrupted Keratinocytes—In cultured cells, integrins and tyrosine-phosphorylated proteins are concentrated at focal adhesions, where actin cytoskeletons are connected with the extracellular matrix. Many lines of evidence have revealed that the number and/or tyrosine phosphorylation status of focal adhesions influence adhesiveness, spreading, and cell migration (10, 11, 14, 23). Therefore, we next examined the formation and tyrosine phosphorylation status of focal adhesions by immunostaining with an anti-phosphotyrosine monoclonal antibody. Strikingly, individual STAT3-disrupted keratinocytes exhibited coarse and strong signals on the surface (Fig. 2, right panel, red arrowheads) compared with control cells (Fig. 2, left panel), indicating that focal adhesions of STAT3-disrupted keratinocytes were hyperphosphorylated.

Tyrosine Phosphorylation Status of STAT3-disrupted Keratinocytes—To determine the focal adhesion molecule responsible for hyperphosphorylation in STAT3(−/−) keratinocytes, Western blot analysis was performed. Whole cell lysates (Fig. 3a, left panel) or PY20 immunoprecipitates (Fig. 3a, right panel) were subjected to Western blot analysis with PY20. Approximately 60–90 and 120–130-kDa proteins were hyperphosphorylated on tyrosine residues in STAT3-disrupted keratinocytes (Fig. 3a, black arrows).
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Each test, although no significant differences were observed at 1 and 3 h.

Bar N.S.

protrusions (black arrows). However, nonspread cells were rounded and flat, representative photographs at 20 min.

b

FIG. 1. STAT3-disrupted keratinocytes exhibit increased adhesiveness and spreading onto the collagen matrix. a, analysis of cell attachment to the collagen matrix. Freshly isolated keratinocytes were seeded onto type I collagen-coated dishes. After incubation for 20 min, 1 h, and 3 h, nonadherent cells were removed by washing, and attached cells were counted. STAT3-disrupted keratinocytes (white bars) showed significantly stronger cell adhesiveness to the collagen matrix than control cells (black bars) at 20 min \((p < 0.05, \text{Student’s } t\) test), although no significant differences were observed at 1 and 3 h. Each \(\text{bar}\) represents the mean ± S.D. of three independent experiments. N.S., not significant. \(b\) and \(c\), analysis of cell spreading on the collagen matrix. Freshly isolated keratinocytes were seeded onto type I collagen-coated dishes, and nonadherent cells were washed out. Photographs were taken using a phase-contrast microscope after incubation for the indicated times. \(b\), representative photographs at 20 min. Spread cells were identified as opaque cells, exhibiting membranous protrusions (black arrows). However, nonspread cells were rounded and phase-bright under a phase-contrast microscope. Bar, 20 \(\mu\)m. \(c\), quantitative evaluation of keratinocyte spreading on the collagen matrix. STAT3-disrupted keratinocytes (white bars) showed higher spreading than control cells (black bars) at 20 min \((p < 0.005, \text{Student’s } t\) test). Each \(\text{bar}\) represents the mean ± S.D. of three independent experiments.

p130cas Was Constitutively Hyperphosphorylated in STAT3-disrupted Keratinocytes—Previous studies showed that many tyrosine-phosphorylated proteins are associated with focal adhesions, including nonreceptor kinases, adaptor proteins, and cytoskeletal proteins (13, 24, 25). Among them, focal adhesion kinase (FAK; \(M_r \sim 125,000\)), p130cas (\(M_r \sim 130,000\)), paxillin (\(M_r \sim 68,000\)), and Src (\(M_r \sim 60,000\)) were candidates for the hyperphosphorylated proteins observed in Fig. 3a. Therefore, we analyzed the tyrosine phosphorylation state of these proteins. As shown in Fig. 3b, no differences were demonstrated in protein expression or tyrosine phosphorylation levels of FAK, Src, and paxillin between control and STAT3-disrupted keratinocytes. However, p130cas was constitutively hyperphosphorylated on tyrosine residues in STAT3(−/−) keratinocytes (Fig. 3b, black arrow). p130cas is thought to be an adaptor protein that contains an Src homology 3 (SH3) domain, a central substrate domain, and proline-rich motifs (26). The central substrate domain has multiple phosphotyrosine motifs where Src homology 2 (SH2) domain-containing molecules recruit. The hyperphosphorylation of p130cas in STAT3-disrupted keratinocytes may result in abnormal assembly of interacting proteins, aberrant turnover of focal adhesions, increased cell adhesiveness, and faster cell spreading, leading to impaired migration. It is possible that the hyperphosphorylation of p130cas might be the result of down-regulation of protein-tyrosine phosphatases.

Protein-tyrosine phosphatase, PTP-PEST, has been shown to interact with p130cas, and it has been demonstrated that PTP-PEST(−/−) fibroblasts have a phenotype similar to STAT3(−/−) keratinocytes (10). Therefore, we examined expression levels of PTP-PEST. However, as shown in Fig. 3c, no difference was demonstrated in protein expression levels of PTP-PEST between control and STAT3(−/−) keratinocytes.

Rescue of the Phenotype of STAT3-disrupted Keratinocytes by Reintroduction of a Wild-type STAT3 Gene—We previously demonstrated that the migration of STAT3-disrupted keratinocytes was impaired (7). Control keratinocytes (STAT3+/−) migrated in response to EGF and HGF (Fig. 4, a (top panels) and b (black bars)). In contrast, the migration of STAT3-disrupted keratinocytes (STAT3(−/−)) was severely compromised (Fig. 4, a (middle panels) and b (white bars)). These findings suggest that STAT3 plays a critical role in keratinocyte migration in response to growth factors that activate STAT3, including EGF and HGF. To clarify whether STAT3 deficiency was primarily responsible for the phenotype, we introduced the wild-type Stat3 gene into STAT3(−/−) keratinocytes.

FIG. 2. STAT3-disrupted keratinocytes exhibit an increased number of hyperphosphorylated focal adhesions. Keratinocytes were immunostained with an anti-phosphotyrosine monoclonal antibody, PY20. Focal adhesions were visible as green spots. STAT3-disrupted keratinocytes exhibited an increased number of hyperphosphorylated focal adhesions (right panel, red arrowheads) compared with control cells (left panel). Bar, 20 \(\mu\)m.
Adenoviral vector. Upon reintroduction of wild-type STAT3, impaired migration of the STAT3-deficient keratinocytes was completely reversed (Fig. 4, a (bottom panels) and b (gray bars)), while an empty vector alone could not rescue the phenotype (data not shown). Furthermore, reexpression of Stat3 using an adenoviral vector normalized the tyrosine phosphorylation level in focal adhesions and p130Cas (Fig. 5, a (right panel) and b (top panel, black arrow)), respectively. These findings indicate that STAT3 abrogation primarily leads to compromised cell migration through hyperphosphorylation of p130Cas.

**DISCUSSION**

We previously reported that cell migration was severely compromised in STAT3(-/-) keratinocytes in vivo and in vitro (7). In the present study, we showed that STAT3(-/-) keratinocytes display enhanced cell adhesion and spreading. Cell migration is a coordinated, multistep processes involving 1) extension of membrane protrusions (filopodia and lamellipodia) at the leading edge, 2) formation of attachment sites to the...
extracellular matrix (focal adhesions) at the newly formed cell periphery, 3) cell traction, which might be driven by contraction of the actin cytoskeleton, and 4) the release of adhesions at the rear portion of the cell (detachment). It has been reported that cell migration depends on adhesiveness to the substratum (27). The authors have examined the relationship between maximum migration speed and cell adhesiveness and demonstrated that maximum migration occurs at an intermediate level of cell adhesiveness. The strong adhesiveness might disturb the detachment step and lead to impaired cell migration. Conversely, abnormality in the breakdown rate of focal adhesions might interfere with the detachment step and lead to an increased number of focal adhesions and strong adhesiveness to the extracellular matrix. This hypothesis is supported by the observation that FAK(−/−) fibroblasts showed an increased number of focal adhesions, strong adhesiveness, and impaired cell migration (23). In FAK(−/−) fibroblasts, no changes in the expression of β1 integrins were found, which is similar to STAT3(−/−) keratinocytes. The authors concluded that FAK might be involved in the turnover of focal adhesion during cell migration but not in the formation of focal adhesions. Considering this, it is possible that the phenotype of STAT3(−/−) keratinocytes might be the result of focal adhesion turnover failure.

The present study also demonstrated that STAT3-disrupted keratinocytes exhibited a constitutive hyperphosphorylation status in the focal adhesion molecule, particularly p130Cas. p130Cas was originally identified as a protein that was highly tyrosine-phosphorylated in v-Src- and v-Crk-transformed cells (28). The role of p130Cas signaling in cell spreading by anchoring the actin cytoskeleton to focal adhesion has been suggested by the fact that myofibrils and Z discs in cardiocytes were disorganized in p130Cas(−/−) mice and that actin stress fiber formation was severely impaired in p130Cas(−/−) primary fibroblasts (29). p130Cas has several protein interaction motifs, including an SH3 domain, a central substrate domain (tyrosine phosphorylation sites), and proline-rich motifs (30). Upon integrin stimulation by means of binding to the extracellular matrix, FAK undergoes autophosphorylation, providing a binding site for nonreceptor tyrosine kinases, such as Src (31, 32). p130Cas binds to the proline-rich sequence of FAK via its SH3 domains (33) and is tyrosine-phosphorylated by FAK and/or Src (25). Tyrosine-phosphorylated p130Cas plays a role as an adapter molecule, where SH2 domain-containing proteins such as Crk are recruited (25, 34). Recent evidence indicates that this p130Cas/Crk coupling serves as a molecular switch for activating small GTPase Rac through the small GTPase-activating protein DOCK180 and leads to the rearrangement of the actin cytoskeleton (15, 34). On the other hand, upon certain extraacellular stimuli, protein-tyrosine phosphatases (PTP-PEST, SHP2) might also be recruited (36, 37), leading to the dephosphorylation of p130Cas and other focal adhesion-associated proteins. These tyrosine phosphorylation-dephosphorylation cycles might contribute to the formation and stability of focal adhesions and reorganization of cytoskeletal proteins. In STAT3-disrupted keratinocytes, p130Cas was hyperphosphorylated on tyrosine residues (Fig. 3b), although the tyrosine phosphorylation levels of the interacting protein kinases, FAK and Src, were normal (Fig. 3b). Although the exact sites of phosphorylation in FAK and Src were not investigated, the normal phosphorylation status found in STAT3(−/−) keratinocytes suggested that the catalytic activities of these kinases were normal. It is therefore possible that dephosphorylation of p130Cas was decreased due to suppression of catalytic activities and/or failure in p130Cas targeting of protein-tyrosine phosphatases. To date, protein-tyrosine phosphatases that have been demonstrated to interact with p130Cas in vivo and/or in vitro are PTP1B, SHP2, and PTP-PEST (36–38). Interestingly, it has been reported that PTP-PEST(−/−) fibroblasts display a phenotype similar to STAT3(−/−) keratinocytes (10). The authors demonstrated that PTP-PEST(−/−) cells had decreased motility and increased focal adhesions and that PTP-PEST(−/−) cells spread faster, while p130Cas was constitutively hyperphosphorylated.

It is still unclear why a deficiency in STAT3 leads to the hyperphosphorylation of p130Cas in keratinocytes. When STAT3(−/−) keratinocytes were reconstituted with wild-type STAT3 using an adenoviral vector, the impaired migration and the tyrosine phosphorylation level in focal adhesions and p130Cas were normalized simultaneously (Figs. 4 and 5). These findings suggest that the STAT3 defect is primarily responsible for the phenotype of STAT3(−/−) keratinocytes. STAT3 is a member of the STATs activated by a variety of cytokines and growth factors such as interleukin-6, EGF, and HGF. In response to cytokine or growth factor signals, STAT3 is phosphorylated on a tyrosine residue (Tyr-705). STAT3 has an SH2 domain for the recognition of proteins involving the aberrant phosphorylation of p130Cas. However, we found no difference in the expression levels of tyrosine kinases (Fig. 3b) or a tyrosine phosphatase, PTP-PEST (Fig. 3c), between control and STAT3(−/−) keratinocytes. It is also possible that STAT3 functions as an adapter molecule, which recruits kinases and/or phosphatases to p130Cas via a tyrosine residue (Tyr-705) or an SH2 domain. Recently, several reports have suggested that STAT3 plays such roles (40, 41).
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Chaturvedi et al. (40) reported that STAT3 interacted with v-Src in myeloid cells. Ryu et al. (41) demonstrated that STAT3 was immunoprecipitated with FAK in neutrophils. Although we could not demonstrate the interaction between STAT3 and these kinases in keratinocytes (data not shown), it remains to be investigated whether STAT3 interacts with other signal-transducing molecules involved in p130Cas phosphorylation status.

In summary, our results suggest that STAT3 in keratinocytes plays an important role in modulating tyrosine phosphorylation of p130Cas and cell adhesiveness to the substratum leading to cell migration.

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