Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization

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Abstract

During epithelial cell migration, membrane ruffles can be visualized by phase contrast microscopy as dark waves arising at the leading edge of lamellipodia that move centripetally toward the main cell body. Despite the common use of the term membrane ruffles, their structure, molecular composition, and the mechanisms leading to their formation remained largely unknown. We show here that membrane ruffles differ from the underlying cell lamella by more densely packed bundles of actin filaments that are enriched in the actin cross-linkers filamin and ezrin, pointing to a specific bundling process based on these cross-linkers. The accumulation of phosphorylated, that is, inactivated, cofilin in membrane ruffles suggests that they are compartments of inhibited actin filament turnover. High Rac1 and low RhoA activities were found under conditions of suboptimal integrin–ligand interaction correlating with low lamellipodia persistence, inefficient migration, and high ruffling rates. Based on these findings, we define membrane ruffles as distinct compartments of specific composition that form as a consequence of inefficient lamellipodia adhesion.

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Introduction

Cell migration is central not only to many biological processes, including embryogenesis, the inflammatory response, tissue repair, and regeneration, but also to pathological processes such as cancer and metastasis. The onset of migration is accompanied by the acquisition of a spatial asymmetry that is manifested in a polarized cell morphology, that is, a clearly distinguishable front and rear of the cell. Cell migration can be viewed as a multistep cycle beginning with the extension of membrane protrusions (lamellipodia, filopodia) at the cell lamella, which is the flat, almost organelle free front region of motile cells. The cycle is continued by the formation of cell substrate adhesions near the tips of protrusions and the acto-myosin powered contraction of the cell body to be finally completed by the release of cell-substrate adhesions at the cell rear [1,2].

The formation of lamellipodia and filopodia at the cell front is driven by the assembly of actin filaments from the cytoplasmic pool of monomeric actin bound to sequestering proteins such as profilin and thymosin β4 [3]. The signals for actin assembly to start are provided by constituents of the extracellular matrix (ECM) such as fibronectin (FN) and by soluble motogens from the large families of growth factors including the epidermal growth factor (EGF) [4,5]. Both kinds of signals are sensed by appropriate transmembrane receptors that, upon ligand binding, activate different signaling pathways [6,7]. The most prominent receptors for ECM components are the proteins of the integrin gene family [6] that, upon activation by ligand binding, can activate the small GTPases of the Rho family such as RhoA, Rac1, and Cdc42 [5,8]. Rac1 and Cdc42 have been shown to act as regulators of actin assembly essential for lamellipodia and filopodia protrusion [9]. Of the many effector proteins that interact with activated Rac1...
and Cdc42, the Wiskott–Aldrich syndrome protein (WASP) and its ubiquitous family member N-WASP provide a direct link to actin assembly by activating the Arp2/3 complex [10]. This complex acts as a nucleation point for the assembly of the branched actin network that is the predominant cytoskeletal element of growing lamellipodia. According to their role as promoters of actin assembly, Arp2/3 and WASP are concentrated at the tips of lamellipodia of migrating cells [11,12].

Since the cell is a bounded compartment, rapid assembly of actin filaments cannot continue for long without being balanced by rapid and highly regulated disassembly. The ubiquitous proteins of the ADF/cofilin family have been shown to bind to actin filaments and, thereby, promote their disassembly [13]. The activity of ADF/cofilin depends on LIM-kinase-controlled phosphorylation that blocks its interactions with actin [14]. Since LIM-kinase is a downstream effector of Rho family GTPases, actin assembly is promoted in two ways: by activating the Arp2/3 nucleation complex and by inhibiting disassembly via ADF/cofilin regulation. Recent findings additionally point to a positive engagement of cofilin in actin assembly by generating free barbed ends that are a prerequisite for filament elongation by the addition of actin monomers [15].

For sustained migration to occur, the newly formed membrane protrusions have to be stabilized by the formation of firm adhesions to the substrate [16]. The initial step during the establishment of these adhesions has been shown to be the aggregation of ligand-bound integrins in the form of small focal contacts (FC) at the leading edge of protrusions. This aggregation of integrins then triggers the activation of signaling molecules such as the small GTPase RhoA that is responsible for the recruitment of cytoskeletal- and FC-associated proteins, resulting in the formation of contractile acto-myosin bundles and the generation of the larger and more organized focal adhesions (FA) [8,9,16,17]. The small FCs at the cell front are thought to be essential for cell locomotion and act as anchoring sites for the transmission of acto-myosin-driven propulsive forces, whereas the larger FAs seem to inhibit cell migration by forming stable and less dynamic anchoring structures [18,19].

Lamellipodia that fail to establish stable adhesions are believed to become detached from the substrate and retracted toward the cell body [6,16,17]. It has been speculated that during this retraction process, membrane ruffles are formed that are clearly visible in the light microscope as waves of dark contrast on the frontal surface of cells. These ruffles emerge at the cell edge, move centripetally, and finally disappear at the border between the cell lamella and the main cell body [20,21]. What still remained largely unknown are their structure and their molecular composition as well as the signals and the mechanisms leading to ruffle formation. We have applied high-resolution scanning and transmission electron microscopy (EM) to reveal the structural organization of membrane ruffles. To gain insight into the process of ruffle formation and the potential reorganization of the actin filament system, we have analyzed their protein composition, particularly in relation to that of lamellipodia. Our observations show that ruffles contain densely packed arrays of actin filaments endowed with a specific set of associated proteins including the cross-linkers filamin and ezrin, and the assembly regulator ADF/cofilin. To elucidate the functional significance of ruffle formation during cell migration, we have quantified simultaneously with highest resolution in space and time cell migration velocity, the frequency of ruffle formation, the persistence of lamellipodia, and their dependence on the adhesive properties of the substrate [20,22]. Our findings show that conditions of suboptimal cell-substrate adhesion that lead to a significantly reduced lamellipodia persistence and an inefficient cell migration result in a dramatic increase in ruffle frequency. Our data point to integrin-dependent and RhoGTPases-mediated signaling pathways controlling lamellipodia protrusion and ruffle dynamics. We conclude that ruffles are formed as a consequence of inefficient integrin–ligand interaction at the leading edge of lamellipodia and that they act as compartments of actin reorganization.

Materials and methods

Cells and cell culture

Normal human epidermal keratinocytes from neonatal foreskin were purchased from Cambrex Bio Science (Verviers, Belgium). Subcultures were grown at 37°C and 5% CO₂ in complete keratinocyte basal medium (Cambrex Bio Science). Cells were harvested for subculturing or for subsequent experiments using 0.025% Trypsin and 0.01% EDTA in HBSS (Cambrex Bio Science) only between passages 2 and 4. For immunofluorescence staining and scanning EM, cells were subconflently plated on glass cover slips or CELLocate™ cover slips (Eppendorf, Hamburg, Germany). For quantification of cell motility, cells were plated into chambered cover slips (Nalge Nunc International, Wiesbaden, Germany). For biochemical measurements of Rac1 and Rho activity, the cells were plated on 10-cm culture plates. All cells were plated on uncoated or FN-coated surfaces (see below). One hour before starting the quantification of cell motility, staining of integrin β1, or performing Rac1 and RhoA activity assays, the cells were stimulated with 50 nM human recombinant EGF (Sigma-Aldrich, Deisenhofen, Germany) to induce cell migration.

Cell adhesion substrates and anti-integrin antibodies

Coating with human FN (Cell-Systems, St. Katharinen, Germany) at concentrations of 2.5, 5, 7.5, or 10 µg/cm² was done as described previously [23]. To inhibit the

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interaction between integrins and their ligands, function-blocking antibodies against integrin α5 (clone P1D6) and β1 (clone JB1A) (Chemicon, Temecula, USA) were used at 10 and 40 μg/ml, respectively, after saturation with 1% BSA for 30 min.

Quantification of cell motility

Phase contrast image series of motile keratinocytes were obtained using an inverted LSM 510 (Zeiss, Oberkochen, Germany) equipped with a 63 × 1.4 NA Ph3 plan apochromat objective and an incubation chamber for constant temperature, and were controlled by the LSM 510 standard software. Lamella dynamics and cell migration velocity were analyzed by the computer-assisted stroboscopic analysis of cell dynamics (SACED) (Fig. 1) that has been described before [20,22] and renamed recently as kymographic assay [24]. For each substrate, at least 15 individual cells were monitored over a 10-min period by capturing digital images every 2 s. Subsequently, four areas of interest were marked on each image by lines that crossed the cell lamella (Fig. 1a). The resulting 1-pixel-wide areas were cut (Fig. 1b) and lined up in time space plots (Fig. 1c) that allowed the quantification of relevant motility data. In these stroboscopic time space plots, membrane ruffles appeared as dark descending lines (Fig. 1c, black line), whereas lamellipodia protrusions were represented by linear ascending contours (Fig. 1c, white line). The slope of these lines corresponds to the velocity of ruffle and lamellipodia movement \( v = \frac{dx}{dt} \) (min)). Projections of these lines along the x-axis (time) were used to calculate the persistence of lamellipodia, that is, the period a protrusion lasts before its retraction begins (Fig. 1c, dashed white line). The number of lines per time was counted to determine the frequency of ruffle formation. Cell migration velocity was determined by exploiting the clear halo that represents the border between the main cell body and the lamella. Marking this halo (Fig. 1c, dashed black line) allowed to calculate the cell body displacement in relation to the substrate, which is a common definition of cell translocation [25].

**Immunofluorescence staining**

For immunofluorescence staining of integrin β1 (mouse monoclonal anti-human integrin β1, clone P5D2, Chemicon), filamin (mouse monoclonal anti-filamin, clone PM6/317, Chemicon), and ezrin (mouse monoclonal anti-ezrin, clone 3C12, Sigma-Aldrich), the cells were fixed in 4% freshly made paraformaldehyde (PFA) in cytoskeleton buffer [26] (150 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 5 mM Glucose, 10 mM MES pH 6.1) for 20 min at 37°C, washed in PBS containing 30 mM Glycin (G-PBS), permeabilized in PBS/0.2% Triton X-100 for 10 min, rinsed in PBS, and blocked in 3% BSA/PBS for 30 min. The samples were incubated with primary antibodies diluted in 0.3% BSA in PBS for 45 min at 37°C, washed three times with PBS, and incubated with goat anti-mouse Cy3-conjugated secondary antibodies (Jackson Laboratories, West Grove, PA, USA) for 45 min at 37°C together with Alexa-488 Phalloidin (Molecular Probes, Leiden, Netherlands) for correlative actin filament staining. Immunofluorescence staining of cofilin and phosphorylated ADF/cofilin was modified from Ref. [27]. The cells were fixed with 3% PFA together with 0.1% (vol/vol) glutaraldehyde in cytoskeleton buffer for 1 h, washed extensively in PBS, and permeabilized with 0.5% Triton X-100 in PBS for 15 min. After three times washing in PBS, once in TBS (20 mM Tris, 145 mM NaCl, pH 8) and twice with 2 mg/ml NaBH4 in TBS for 15 min, the samples were rinsed four times in PBS and blocked in 3% BSA/PBS for 30 min. Cells were further incubated for 45 min at 37°C with anti-cofilin antibody (rabbit polyclonal anti-cofilin, Cytoskeleton Inc., Denver, USA) and anti-phospho ADF/cofilin antibody (rabbit antiserum raised against a phosphopeptide epitope of ADF/cofilin [28], gift from J.R. Bamburg, Colorado State University, USA), respectively, followed by incubation with a Cy2-conjugated anti-rabbit antibody and Alexa546 Phalloidin for 1 h at 37°C. All samples were mounted in anti-fade reagent (Biomedia Corporation, Foster City, CA, USA) and analyzed by using a LSM 510 equipped with a 63 × NA.
1.4 plan apochromat objective. All images were obtained as confocal z stacks spanning the whole cell in its z range with subsequent projection of the image series into a single overlay using the LSM 510 standard software. For fluorescence quantification using Image-Pro Plus 5 (Media Cybernetics), staining conditions and microscope settings were identical for all samples. All measurements were made on unprocessed images. In brief, the total fluorescence of an area of interest (sum of all gray levels) marking membrane ruffles or lamellipodia was measured and normalized to the size of the respective area. On this basis, the fluorescence ratios of membrane ruffles versus lamellipodia from five individual cells were calculated for actin/filamin and for actin/ezrin.

**Correlative light and electron microscopy**

Cells grown on CELLocate™ cover slips were labeled against actin filaments using Alexa546-Phalloidin (Molecular Probes) as described above. After light microscopic analysis, the cells were treated as described below for scanning EM.

**Scanning electron microscopy**

For scanning EM, cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3, for 20 min, transferred to 0.1% aqueous tannic acid, and rinsed with distilled water. For scanning EM of the cytoskeleton, the cell membrane was removed by detergent lysis. In detail, cells were incubated in PEG-GTX (10 mM PIPES, 50 mM EDTA, 10 mM KOH, 27 mM KCl, 0.1% (vol/vol) Triton X-100, 4% (vol/vol) polyethyleneglycol (MW 6000), 10% (vol/vol) glycerin, pH 7.2) for 5 min, washed briefly in PBS, and fixed with 4% (vol/vol) glutaraldehyde in PBS. All specimens were dehydrated through a graded series of ethanol and critical point dried from CO2 in 10 cycles according to Ref. [29] using a Balzers CPD 030 (BAL-TEC, Schalksmühlen, Germany). Dried specimens were mounted on aluminum sample holders and coated with a 2-nm layer of platinum/palladium in a HR 208 sputter coating device (Cressington, Watford, UK). SEM was performed at an acceleration voltage of 3 kV using a XL 30 SFEG (Philips, Eindhoven, Netherlands) equipped with a through lens secondary electron detector.

**Rac1 and rhoA activity assays**

Many Rho family effector proteins specifically recognize the activated, GTP-bound form of RhoA and Rac1. We used PAK-1- and Rhotekin-coupled agarose beads (Cytoskeleton) to precipitate PAK-1/RacGTP- and Rhotekin/RhoGTP complexes that can be identified in Western blots using anti-Rac and anti-Rho antibodies. In brief, keratinocytes grown on 10-cm culture plates or FN-coated 10-cm culture plates were stimulated with 50 nM EGF for 1 h and lysed with lysis buffer (50 mM TRIS, 0.5 M NaCl, 1% Triton X-100, 10 mM MgCl2, pH 7.5). After addition of agarose beads to the cell lysates and incubation at 4°C for 60 min, the agarose beads were washed and subsequently collected by centrifugation at 5000 g. The beads were then resuspended in Laemmli-buffer, separated by SDS-PAGE, and detected by Western blot using rabbit polyclonal anti-Rac and mouse monoclonal anti-Rho antibodies (Cytoskeleton) and appropriate secondary HRP-conjugated antibodies. Staining was detected using enhanced chemiluminescence and densitometry has been done using Optiquant (Packard Instruments, Meridan, USA).

**Statistical analysis**

Statistical evaluation of SACED-derived motility data was performed with SPSS software (SPSS Inc., Chicago, USA). Results were expressed as mean values with bars representing 95% confidence interval of mean values. Statistical significance between data groups was determined by Whitney U test and considered to be significantly different at values $P < 0.01$. Data were tested for linear regression and rank correlation was determined according to Spearman and considered to have statistical significance at values $P < 0.01$.

**Results**

**Ruffles are flat membrane folds on the lamella surface**

In phase contrast microscopy of motile keratinocytes, membrane ruffles were clearly visible as waves of dark contrast that move centripetally from the leading edge of the organelle free cell lamella toward the organelle containing main cell body where they finally disappeared. Cells grown on not precoated glass surfaces migrated inefficiently (about 0.2 μm/min; see below) and exhibited numerous ruffles evenly distributed over the cell lamella (Fig. 2a, arrows), whereas cells on FN-coated surfaces migrated efficiently (about 0.5 μm/min; see below) and bore only few if any ruffles (Fig. 2b). Scanning EM revealed a narrow, fold-bearing lamella for cells grown on not precoated glass surfaces (Fig. 2c) and an extended, smooth lamella for cells grown on FN (Fig. 2d). At higher magnifications, ruffles appeared as flat membrane folds that protruded from the lamella resembling waves on a water surface (Fig. 2e, arrows). Underneath these membrane folds newly formed membrane protrusions were observed (Fig. 2e, arrowheads). Time-lapse studies of the cell lamella allowed showing that lamellipodia detachment and their subsequent retraction toward the cell body are the main steps of ruffle formation (Figs. 3a and b). Analysis of cells grown on microgrid cover slips revealed that the dark waves on the cell lamella visualized by phase
contrast microscopy (Fig. 3c, arrows) indeed corresponded to the extended membrane folds observed by scanning EM (Fig. 3d, arrows).

Membrane ruffles contain densely packed actin filaments with a characteristic set of associated proteins

Scanning EM of the keratinocyte lamella after removal of the plasma membrane by detergents revealed that ruffles contained densely packed arrays of thin filaments (Fig. 3f, arrows), which were only sparsely distributed in the underlying lamella. Fluorescence quantification revealed a 50% higher density of actin filaments within membrane ruffles as compared to lamellipodia. This is consistent with the dense packaging of actin filaments observed by SEM (Fig. 3f). An even higher increase in the concentration of ezrin by 150% and of filamin by 100% was measured. This corresponds to an about 2-fold increase in the filamin/actin and an about 3-fold increase in the ezrin/actin ratio in membrane ruffles as compared to lamellipodia and points to a specific recruitment of both proteins during membrane ruffle formation and concomitant actin filament reorganization. Intriguingly, the actin filament severing protein ADF/cofilin was found in ruffles exclusively in its phosphorylated, that is, inactive, form (Figs. 3e and f, circle), whereas the
activated form was evenly distributed across the lamella region (Figs. 3g and h).

The SACED motility assay allows to quantify the dynamics of ruffles and lamellipodia in relation to migration velocity

The SACED cell motility assay-derived stroboscopic time space plots allowed to simultaneously visualize the dynamics of membrane ruffles, which appeared as dark descending lines (Figs. 5a and e, black lines), and of lamellipodia, which represented linear ascending contours (Figs. 5a and e, white lines). The slope of these lines corresponded to the velocity of ruffle and lamellipodia movement and projections of these lines along the x-axis (time) allowed to calculate the persistence of lamellipodia, that is, the period a protrusion lasts before its retraction begins (Fig. 5a, dashed white line). The number of lines per time was counted to determine the frequency of ruffle formation. In addition, cell migration velocity was determined by exploiting the clear halo, which represents the border between the main cell body and the lamella. Marking this halo (Fig. 5a, dashed black line) allowed to calculate the cell body displacement in relation to the substrate that is a common definition of cell translocation [1].

High ruffle frequency indicates low lamellipodia persistence and inefficient cell migration

Evaluation of the SACED-derived motility data showed that migration velocity reached a minimum of about 0.21 (±0.18) μm/min on not precoated glass surfaces (Fig. 5b), whereas significantly higher velocities occurred on FN-coated surfaces with a maximum of about 0.53 (±0.3) μm/min at 2.5 μg/cm² FN. With increasing FN concentrations, the migration velocity dropped in a linear fashion to only 0.24 μm/min at 10 μg/cm² FN. Lamellipodia persistence reached lowest values on not precoated glass surfaces (Fig. 5c) with an average life time of about 0.37 (±0.15) min, whereas at 2.5 μg/cm² FN, a nearly 10-fold higher persistence of about 3.34 (±1.33) min was determined.

As for migration velocity, the persistence of lamellipodia decreased in a linear relation with increasing FN concentrations dropping to about 0.6 min at 10 μg/cm² FN. Ruffle frequencies reached maximum values on not precoated glass surfaces with about 0.2 (±0.08) ruffles/min (Fig. 5d), whereas lowest ruffle frequencies were measured with about 0.06 (±0.04)/min at 2.5 μg/cm² FN. With increasing FN concentrations, a linear rise in ruffle frequencies occurred up to 0.14 (±0.06)/min at 10 μg/cm². All data point to a clear correlation between migration velocity, that is, the efficiency of migration, the persistence of lamellipodia, and the frequency of membrane ruffle formation. Under substrate conditions that supported the formation of highly persistent lamellipodia, efficient migration occurred and ruffling became less frequent. Under conditions favoring short-lived lamellipodia, migration was slow, that is, inefficient, and ruffle formation was more frequent. Hence, membrane ruffles might be considered as indicators of inefficient migration due to reduced lamellipodia persistence.

Lamellipodia persistence and ruffle formation depend on the interaction of integrin α5β1 with its ligand FN

Newly formed lamellipodia are known to be stabilized by the interaction of integrins with their ECM ligands [16]. After addition of antibodies blocking the interaction of the integrins α5 and β1 with FN, we observed a significantly reduced migration velocity of keratinocytes dropping by 75% to about 0.08 (±0.04) μm/min for integrin β1 (Fig. 5f). Simultaneously, lamellipodia persistence decreased to 0.037 (±0.15) min thus reaching only 1% of control values (Fig. 5g), whereas ruffle formation became dramatically more frequent reaching 5-fold values of controls (Fig.
Incubation with 1% BSA had no effect on cell motility (not shown). Using function-blocking antibodies against various other integrins expressed by migrating keratinocytes caused no significant effects on the motility parameters described above (not shown). Our data indicate that integrin α5β1 interaction with FN is essential for the attachment of lamellipodia leading to high lamellipodia persistence and allowing efficient cell migration. Hence, the formation of ruffles is apparently a consequence of a suboptimal interaction between integrin α5β1 and its ligand FN.

Efficient migration depends on low rac and high rho activity

The small GTPases Rac1 and RhoA belonging to the Rho-family are known to be involved in the downstream
signaling of ligand-bound integrins. We applied Rac1 and RhoA activity assays to determine the relationship between GTPase activity and cell migration efficiency. Rac1 activity was low for efficiently migrating keratinocytes exhibiting low ruffle frequencies and highly persistent lamellipodia at 2.5 μg/cm² FN (Fig. 6a). Rac1 activity was found to be significantly increased when cells were grown on high FN concentrations or on not precoated glass surfaces, that is, on substrates that allowed only reduced migration efficiency accompanied by diminished lamellipodia persistence and frequent ruffle formation. Highest Rac1 activity was found at 10 μg/cm² reaching about 3-fold higher levels as compared to 2.5 μg/cm² FN. About 2.5-fold higher levels of Rac1 activity were also measured for cells grown on not precoated glass surfaces. Contrary to Rac1, RhoA showed highest activity levels in cells grown on 2.5 μg/cm² dropping significantly on higher FN concentrations to only 40% on 10 μg/cm² and to 60% for cells grown on not precoated glass surfaces (Fig. 6b). Hence, high RhoA and low Rac1 activities coincided with efficient cell migration, that is, fast cell movement, high lamellipodia persistence, and low ruffling frequencies.

Formation of stress fibers and focal adhesions are characteristics of inefficiently migrating cells

Downstream effectors of the Rho GTPase are various actin-associated proteins that modulate the formation of contractile acto-myosin fibers and the clustering of integrin β1 [17] in the form of FC at the front of lamellipodia that are required to convert contraction into traction forces necessary for cell migration [18]. Cells grown on not precoated glass surfaces, which have been shown to contain low levels of activated RhoA and high levels of Rac1, were enriched with numerous densely packed bundles of actin filaments (Figs. 7b and c, arrows) and showed a nearly even distribution of integrin β1 but no obvious clustering at the lamella region (Fig. 7a). Cells grown on 2.5 μg/cm² FN, which exhibited the highest levels of RhoA activity and low levels of Rac1 activity, contained only few and significantly smaller bundles of actin filaments (Figs. 7e and f), and integrin β1 was clustered at the cell lamella in the form of distinct patches (Fig. 7d, circles) with a size of about 2 μm that is typical for FCs [30]. Very fine and short bundles of actin filaments were observed that were orientated along the direction of cell migration (Fig. 7e, circles) and were linked to the putative FC sites (Fig. 7f, circles). With increasing FN concentrations, we observed significantly more and thicker bundles of actin filaments representing stress fibers (Fig. 7h, arrows) and larger but less distinct integrin β1 patches (Fig. 7g).

Discussion

The protrusion of lamellipodia at the cell front is regarded as the initial step of a cyclic process that underlies cell migration [1,2]. For efficient migration to occur, lamellipodia have to be stabilized by the formation of cell substrate adhesions that represent molecular bridges by the heterodimeric transmembrane receptors of the integrin family connecting the cytoskeleton and the ECM [16]. Depending on the adhesiveness of the substrate, the formation of adhesions can fail, and it has been speculated that lamellipodia without appropriate anchorage become detached and are retracted from the leading lamella centripetally toward the main cell body [17], thereby forming characteristic structures on the cell surface that are generally described as membrane ruffles [20,21].

To validate the assumed relationship between lamellipodia adhesion and ruffle formation, we applied the high-resolution SACED cell motility assay [20,22,23] that allows to acquire and quantify various parameters of cell migration and motility simultaneously, and has been successfully used to characterize the motogenic activity of growth factors on epidermal keratinocytes and melanocytes [22,31]. To learn more about the signals and mechanisms involved in ruffle formation, we investigated the structural organization and the protein composition of membrane ruffles, and analyzed
the activities of the small GTPases Rac and Rho that are known as downstream effectors of integrin signaling and as mediators of cytoskeletal and adhesive structures formation.

**Ruffling is a function of lamellipodia persistence and directly related to migration efficiency**

FN is one of the major constituents of the dermal ECM and has been shown to represent the preferred migration substrate for keratinocytes during the process of epidermal wound healing [32]. Surfaces coated with FN have been observed to be appropriate for efficient migration of keratinocytes in vitro. In the absence of motogenic growth factors, a saturable increase in migration velocity has been shown in relation to FN concentration for keratinocytes [23], whereas a biphasic relation between migration velocity and the adhesiveness to substrates has been described for CHO [33]. In contrast, this report shows that keratinocytes exhibit this biphasic relation only after EGF stimulation with maximal values occurring at intermediate FN levels, which has also been reported for NR6 fibroblasts [34].

Lower FN concentrations failed to support efficient cell migration, which might be due to insufficient cell–matrix adhesion at the cell front that in turn is essential for the generation of traction forces during cell migration [18]. On FN concentrations above 5 μg/cm², we observed a significant decrease of migration velocity, which is considered to be a consequence of insufficient detachment of adhesion sites at the cell rear [35].

A clear relationship between the application of different motogenic growth factors, the frequency of ruffle formation, and cell migration velocity has been reported [20,23]. Accordingly, the formation of membrane ruffles is usually considered as a sign of increased lamella dynamics and of elevated migration levels [36]. To address the question whether membrane ruffles result from lamellipodia that have failed to form firm attachments, we measured the persistence of lamellipodia, that is, the period a lamellipodium exists before it is retracted, which we found to be a sensitive, rate-limiting, and adhesion-dependent motility parameter. Highly persistent lamellipodia were found to be characteristic of efficiently, that is, fast moving, cells on appropriate FN substrates and to be correlated in all cases with minimal ruffle frequencies. Reciprocal proportions were observed for inefficiently migrating cells that generated only short-lived lamellipodia accompanied by high ruffling frequencies. A correlation between lamellipodia persistence and cell migration efficiency has also been reported recently for fibroblasts [24] without addressing directly the relationship with ruffle formation. Our findings provide for the first time clear evidence that ruffles originate in lamellipodia that fail to properly attach to the substrate and are retracted toward the main cell body. Thus, membrane ruffle formation can be looked upon as a specific
but indirect criterion for the evaluation of lamellipodia persistence, which depends on substrate adhesiveness and is directly related to cell migration efficiency (Fig. 8).

**Lamellipodia persistence depends on the specific interaction between integrin α5β1 and FN**

Cell-substrate adhesion is mainly accomplished by the interaction of ECM receptors from the integrin family and is rate limiting for efficient cell migration [1], and any disturbance of the ECM–integrin interaction has inhibitory effects on cell migration [37]. Keratinocytes migrating on FN use integrin α5β1 for the interaction with the substrate, whereas integrin α3β1 is responsible for dynamic interactions with laminin 5 [38]. Antibodies that block the interaction of the integrins α5 and β1 have been shown recently to significantly inhibit keratinocyte migration velocity on FN [37,39], whereas inhibition of integrin α3 had measurable effects on migration of mammary carcinoma cells on laminin 5 [40]. Our SACED assay-derived data on keratinocytes show that inhibition of the FN receptor components integrin α5 and β1 causes a significant decrease in lamellipodia persistence and migration velocity, whereas ruffle formation becomes dramatically more frequent. The FN-binding integrin α5β1 has been shown previously to be essential for the formation of FC at the front of newly generated lamellipodia [41], which, besides anchoring the lamellipodia, are indispensable for the transformation of cellular contraction forces into traction forces, that is, cell body translocation during migration [18]. Our findings show that small but very distinct integrin clusters characteristic of FC are formed at the lamellipodia front of efficiently migrating cells, but absent from the lamella of inefficiently migrating cells. The interaction of integrin α5β1 with FN can be considered the molecular anchor, the stability of which decides whether lamellipodia are stabilized and, therefore, contribute to efficient cell migration or whether they are retracted as ruffles.

**Ruffle formation is regulated by the small GTPases rac and rho**

Besides acting as molecular linkers between FN and the actin filament system, integrin β1 can activate GTPases of the Rho family following ligand binding [42,43]. Adhesion of keratinocytes to laminin 5 via the integrins α3β1 and α5β4 leads to increased levels of activated Rho [44], and the adhesion of untransformed NIH-3T3 cells on FN specifically enhances RhoA activity [45]. Reexpression of integrin β1 in β1-deficient epithelial cells triggered the activation of both RhoA and Rac1, which raises the possibility that integrin β1 might regulate the motility of epithelial cells by the use of this small GTPases [46]. In mice, loss of integrin β1 caused a severe defect in epidermal wound healing and β1-null keratinocytes showed impaired migration [47]. An increase in RhoA activity occurred after prolonged contact to FN, although an initial decrease was observed upon plating NIH3T3-fibroblasts on FN [48]. RhoA activity, which has been found to antagonize lamellipodia formation [49,50] also, stimulates cellular contractility as a prerequisite for cell migration [51]. RhoA activity, therefore, might be a prerequisite for sustained cell migration under appropriate substrate conditions by regulating cellular contractility and focal adhesions. Our findings on keratinocytes show that high Rho activity coincides with the formation of highly persistent lamellipodia, efficient cell migration, and low ruffling rates. Under these conditions, we observed the formation of very faint bundles of actin filaments terminating in FC sites. It has been demonstrated that 3–10 actin filaments are sufficient to support the forces applied to individual FC [52]. Hence, these bundles of actin filaments might represent the contractile structures responsible for cell body contraction during keratinocyte migration (see Figs. 7d–f).

Upon activation by ligand-bound integrins and growth factor receptors, the GTPase Rac1 triggers the activation of the Arp2/3 complex, resulting in increased assembly of actin filaments, thereby promoting lamellipodia protrusion [10,16,17]. Expression of a constitutively active form of Rac1 showed that activation of this GTPase induces the formation of lamellipodia and ruffling but is not sufficient to propagate cell movement [53,54]. Accordingly, Rac1-deficient macrophages show normal migration and chemotaxis but reduced membrane ruffling in response to colony stimulating factor-1 [55]. Our findings that highest Rac1 activity is observed under conditions of inefficient migration, lamellipodia persistence, and high ruffling rates support the view that Rac1 triggers the protrusion of lamellipodia. Without appropriate adhesion these lamellipodia are not able to support forward movement and are quickly retracted as membrane ruffles. Membrane ruffles are

![Fig. 8. Schematic illustration summarizing the correlation between adhesion-dependent lamellipodia persistence, migration efficiency, and ruffling rates. High ruffling rates indicate high Rac-activity, suboptimal adhesion, and low persistence of lamellipodia that results in inefficient migration. Low ruffling rates point to high Rho activity, adequate adhesion, and highly persistent lamellipodia that result in efficient cell migration.](image-url)
apparently an appropriate structural indicator for Rac1 activity pointing to suboptimal lamellipodia adhesion and inefficient cell migration (Fig. 8).

Filamin and ezrin might act as organizers and stabilizers of membrane ruffles

The actin-associated protein filamin is known as an actin filament cross-linker that is able to form either three-dimensional orthogonal networks when the filamin/actin ratio is about 1:150 or parallel bundles when the ratio is about 1:10 [56]. Our observations showing that the filamin/actin ratio in ruffles is about 2-fold higher than in lamellipodia point to filamin as the bundling protein responsible for the dense packaging of actin filaments observed in membrane ruffles. These results imply that membrane ruffle formation is accompanied by compaction of actin filaments. Besides its cross-linking function, filamin has been shown to be mechanically linked to integrins [57], thereby providing a link between the plasma membrane and the actin filament system that might be necessary for the stability of membrane ruffles during their retraction. Accordingly, filamin-deficient human melanoma cells are still capable to form protrusions upon serum stimulation, but the protrusions form blebs and ruffling is not observed [58,59]. Finally, filamin has been shown to be integrated in cellular signaling pathways that use Rho GTPases as mediators and lead to a phosphorylation-dependent modulation of the binding capacity of filamin for actin [60]. The activity levels of Rac1 and RhoA observed in migrating keratinocytes might represent a direct switch for filamin to preferentially stabilize actin filament bundles as found in membrane ruffles. Ezrin is another prominent actin binding protein known to tether actin filaments to the plasma membrane by its interaction with the hyaluron receptor CD 44 [61]. Functional ablation of ezrin has been shown to block ruffling and migration of fibroblasts [62]. Our finding that the ezrin/actin ratio in ruffles is 3-fold higher than in lamellipodia provides further support for its suggested role during the formation and stabilization of membrane ruffles. The actin filament severing protein ADF/cofilin has been shown to produce free actin monomers that are necessary for the Arp2/3-dependent assembly of actin filaments, which power lamellipodia protrusion [3]. Recent findings additionally point to a positive engagement of cofilin in actin assembly by generating free barbed ends that are a prerequisite for filament elongation by the addition of actin monomers [15].

Upon phosphorylation by the Rho effector kinase LIM, ADF/cofilin becomes inactivated [63]. Our observation that ruffles contain high amounts of phosphorylated cofilin points to ruffles as a compartment where the Arp2/3- and Cofilin-dependent actin filament turnover that powers lamellipodia protrusion becomes inactivated during membrane ruffle formation. The specific protein composition of ruffles and their characteristic arrays of actin filaments suggest that membrane ruffles might act as a compartment involved in the highly regulated reorganization of actin filaments that become necessary when lamellipodia are retracted due to impaired adhesion.

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