Release of integrin macroaggregates as a mechanism of rear detachment during keratinocyte migration

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Introduction

The acquisition of a spatial asymmetry by the cell in order to turn intracellularly generated forces into a net cell body translocation is an essential requirement for cell migration. Maintenance of this asymmetry is achieved by the formation of specialized cell-substrate contact structures that have been described as focal adhesions or hemidesmosomes. Focal adhesions provide the structural linkage between the extracellular matrix (ECM) and the microfilament system, whereas hemidesmosomes link the ECM to the intermediate filaments (Jones and Green, 1991; Burridge et al., 1988). In both cases the linkage between cell and ECM is established by low-affinity transmembrane glycoprotein adhesion receptors including members of the heterodimeric integrin family. The specific integrins that are clustered within focal adhesions are determined by the nature of the ECM adsorbed onto the surface on which cells are growing, e.g. the α5β1 integrin known as the fibronectin receptor (Fath et al., 1989). The principal component for integrating the cytoskeleton and ECM at the site of the hemidesmosome is the α6β4 integrin, also known as the laminin-5 receptor (Jones and Green, 1991; Green and Jones, 1996; Borradori and Sonnenberg, 1999). While hemidesmosomes function in the more long-lasting anchorage of epithelial cells (Jones et al., 1998), focal adhesions are relatively short lived (Kaverina et al., 2002). Both types of cell-substrate contacts are absent or only transiently identifiable during cell migration (Dunlevy and Couchman, 1993; Matsumoto et al., 1994). For a net cell body translocation an asymmetric detachment of these contacts is required, i.e. the detachment of adhesions at the cell rear with maintenance and the formation of new adhesions at the cell front (Lauffenburger and Horwitz, 1996).

Breakage of cell-substratum attachment needed to allow locomotion can, in principle, occur either by intracellular disruption of the cytoskeleton-integrin linkage or by extracellular release of the integrin-matrix linkage. The kinetic model for integrin-mediated adhesion release during migration predicts two distinct detachment phenomena integrating the biochemical and biophysical interactions between integrins,

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the cytoskeleton and the ECM that affect rear retraction and linkage dissociation mechanisms (Palecek et al., 1999). In the first, detachment is extremely rapid, dominated by integrin-ECM dissociation and it occurs at high forces or low adhesive-ness. In the second, detachment is much slower, dominated by integrin-cytoskeleton dissociation occurring at low forces and high adhesiveness (DiMilla et al., 1991; Huttenlocher et al., 1995; Sheetz, 1994). Dissociation of adhesion sites depends on highly regulated events that are mediated by mechanical contraction forces but also by a variety of tyrosine kinases and phosphatases that modulate the affinity of integrins for their ECM ligands and their cytosolic adaptor proteins as well as proteases cleaving the adhesion complex including the cytosolic cytosine protease calpain, matrix proteases and sheddases of the ADAM family.

Originally, these biochemically regulated processes were thought to facilitate rear detachment of migrating cells by a process which does not induce any loss of cell material during migration. However, it has been shown that “membrane ripping” occurs during rear detachment of migrating cells (Bard and Hay, 1975; Chen, 1981). By this process, a major fraction of cell material can remain on the substrate in form of characteristic migration tracks, that have been described for a number of slowly migrating cell types including fibroblasts of different organisms (for review see (Lauffenburger and Horwitz, 1996)) and primary chondrocytes (Zimmermann et al., 1999, 2001). In addition, an apparently slightly different form of cell fragments has been reported for HT-1080 fibrosarcoma cells and MDA-MB-231 mammary carcinoma cells (Woll et al., 2003) migrating through 3D networks of collagen. Migration tracks have been shown to contain high amounts of integrins left behind by chick fibroblasts in form of macroaggregates, i.e., small integrin-containing membranous patches which are left behind attached to the substratum (Regen and Horwitz, 1992). Detailed studies have revealed that cells such as neutrophils or keratinocytes, move more efficiently and more persistently than fibroblasts (Lee et al., 1993), and it has been speculated that such cells do not expend vast quantities of integrins as in contrast observed in fibroblast migration (Palecek et al., 1996). However, this speculation has been questioned by the observation that efficiently migrating epidermal keratinocytes leave behind considerable amounts of their integrins in form of macroaggregates connected to an underlying fibrous meshwork of specific ECM proteins (Kirfel et al., 2003).

In this report, we show that during rear detachment migrating keratinocytes leave behind characteristic tracks that contain macroaggregates, i.e., cellular remnants with a specific set of protein components. These macroaggregates can be distinguished by their size, origin and distribution within the migration track and by their protein composition: type I macroaggregates that apparently stem from former focal adhesion sites, thus containing high amounts of β1 integrin and a variety of associated α-integrins, and type II macroaggregates that obviously derive from fragmentation of hemidesmosomes due to their content of β4 integrins. Moreover we found that macroaggregates are completely membrane-covered compartments which are stable over a long period of time. Due to our findings on composition and structure of these integrin-containing compartments we conclude that the release of macroaggregates occurs by a distinct cellular mechanism of rear detachment, which implies the loss of adhesive receptors of both, focal contacts and hemidesmosomes, as part of membrane-covered cellular remnants.

Materials and methods

Cells and cell culture

Normal human epidermal keratinocytes from neonatal skin were provided by Cambrex Bio Science (Verviers, Belgium) and initiated into culture by a modification of the method described by Rheinwald and Green (1975) and Green et al. (1979). Subcultures were grown at 37°C and 5% CO2 in complete keratinocyte basal medium (KB M) (Cambrex Bio Science). Cells were harvested for sub-culturing or subsequent experiments using 0.025% trypsin and 0.01% EDTA in HBSS (Cambrex Bio Science) only between passages 2–4. To initiate keratinocyte migration, 50 nM EGF was added to the culture medium.

Fluorescence microscopy

Keratinocytes grown for 24 hours in KBM 153 medium with 50 nM EGF on uncoated glass coverslips, or on coverslips coated with 50 μg/ml fibronectin were fixed with 2% paraformaldehyde in PBS for 20 minutes. For immuno-labeling, cells were blocked with 3% BSA in PBS for 10 min either after permeabilization with 0.1% Triton X-100 in PBS or without detergent treatment and incubated with primary antibodies as follows: For integrin-labeling we used monoclonal antibodies against human α3, α5, β1 and β4 integrin (Chemicon, Temecula, USA), diluted 1:100 in PBS. For labeling of cytoskeletal proteins we fixed and permeabilized the cells as described above and applied monoclonal primary antibodies against ezrin, vinculin, cyto keratin 5 and 14 (Chemicon), BP230 (gift from Dr. Stanley, Chicago), and plectin (gift from Dr. Herrmann, Heidelberg), at dilutions between 1:50 and 1:100 in PBS. After incubation for 60 minutes with primary antibodies at room temperature, and washing in PBS cells were incubated with secondary DTAF-conjugated goat anti-mouse or goat anti-rabbit antibodies (Dianova, Hamburg, Germany) diluted 1:100 in PBS for 30 minutes at room temperature. Labeled cells were analyzed using an LSM 510 (Zeiss, Oberkochen, Germany) equipped with an argon laser (488 nm line).

Calcein-AM staining and merocyanine 540 labeling

Calcein AM is a widely used green fluorescent cell marker that is membrane-permeable and thus can be introduced into cells via incubation. Once inside the cells, calcein AM is hydrolyzed by endogenous esterase into the highly negatively charged green fluo- rescent calcein, which is retained in the cytoplasm. Calcein AM is an excellent tool for the studies of cell membrane integrity and for long-term cell tracing. Keratinocytes were labeled prior to plating with the membrane permeable dye Calcein AM (Biotium, Hayward, USA) for 1–30 min at a final concentration of 0.4 μM. To detect lipid components of macroaggregates, merocyanine 540 (Sigma, Taufkirchen, Germany) was added to keratinocytes at a final concentration of 7.5 μg/ml in PBS and incubated for 5 min. Merocyanine 540 is a fluorescent lipophilic probe that binds preferentially to membrane lipids. Labeled cells were analyzed using an LSM 510 (Zeiss) equipped with an argon laser (488 nm line) and helium neon laser (543 nm line).

Scanning electron microscopy (SEM)

Keratinocytes were grown for 24 hours in KBM medium with 50 nM EGF either on uncoated cover slips or cover slips coated with human fibronectin (50 μg/ml). All cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3, for 20 minutes and transferred to 0.1% aqueous tannic acid. After rinsing with distilled water specimens were dehydrated through a graded series of ethanol and critical point dried with CO2 in 10 cycles according to Svitkina et al. (1984) using a Balzers Balatome II (Balzers, Liechtenstein). Specimens were mounted on aluminum sample holders and sputter-coated with 2 nm platinum/palladium in an HR 208 coating device (Cressington, Watford, UK). SEM was performed at an acceleration voltage of 3 keV using an XL 30 SFEG (Philips, Eindhoven, The Netherlands) equipped with a through-lens secondary electron detector.
Transmission electron microscopy (TEM)
Keratinocytes grown on pioloform-covered and fibronectin-coated nickel grids (100 mesh) were fixed in 2% paraformaldehyde in PBS and blocked with 3% BSA in PBS either after permeabilization with 0.1% Triton X-100 in PBS or without detergent treatment. Blocked cells were immuno-labeled with primary antibodies against β1 or β4 integrin as described above for fluorescence microscopy. After washing with PBS cells were incubated with gold-conjugated secondary antibodies (Dianova). After rinsing with distilled water specimens were dehydrated through a graded series of ethanol and critical point dried from CO₂ as described above. Dried specimens were coated with a 2-nm layer of carbon for TEM using a Balzers BAE-080 evaporation device (BALTEC). TEM was performed at an acceleration voltage of 80 kV using a CM 120 electron microscope (Philips) equipped with a LaB₆ filament.

Quantification of integrin loss
Quantification of integrin loss was performed on the basis of LSM images using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). The total length of migration tracks was measured from the tips of retracting fibers to the end of the tracks. Values were determined for cells grown in the presence of 50 nM EGF for 24 hours to calculate the average velocity of migration track formation and migration velocity. To correlate migration velocity and the amount of released integrin cells were classified according to the length of their migration tracks after 24 hours of cultivation on fibronectin as slow (track length < 35 μm), intermediate (35 to 80 μm) and fast (>80 μm). Then, the amount of integrin released within 24 hours of migration on fibronectin was determined by measuring the intensity of fluorescence emission in the migration track and calculation as percent of total intensity of emission, i.e. the emission of the migration track plus keratinocyte cell body emission.

Statistical analysis
All migration assays were repeated at least three times and results were expressed as the mean ± standard error. For statistical differences data were analyzed by means of Student’s t test. Differences from controls were considered to have statistical significance at values p < 0.01. Rank correlation was determined according to Spearman and considered to have statistical significance at values p < 0.01.

Results
Morphology of the migration track – two types of macroaggregates
Transmission EM of migrating keratinocytes revealed the formation of long tubular extensions, the retracting fibers, at the rear of the cell (Fig. 1a). Pointing away from the cell rear and exactly marking the way the cell has taken, characteristic migration tracks were observed (Fig. 1a–e). At higher magnifications the composition of these migration tracks became evident, and two types of structures, the macroaggregates, could be classified due to their size and pattern of distribution (Fig. 1b, c). Type I macroaggregates were spherical and tubular structures with a diameter of about 50–100 nm that were arranged like “pears on a string” and seemed to derive from fragmentation of retracting fibers. Type II macroaggregates were spherical structures with a diameter of about 30–50 nm that were arranged in clusters scattered about the gaps between type I macroaggregates (Fig. 1c, yellow labeling). Scanning EM showed that type II macroaggregates obviously result from membrane rapping at the interspaces between adjacent retracting fibers (Fig. 1d, e).

Integrins as specific markers for type I and II macroaggregates
As shown before, keratinocytes leave behind considerable amounts of their β1 integrin during migration on a variety of different surfaces. Immunofluorescence labeling of migration tracks with antibodies against β1 resulted in a characteristic labeling pattern resembling the “pearl on a string” arrays of type I macroaggregates (Fig. 2a). This could be validated by immuno-gold labeling showing considerable amounts of β1 integrin on the surface of type I macroaggregates (Fig. 2c). We could also show that in addition to β1 integrin several α integrins such as α5 that is known to associate with β1 to form the hemidesmosomal fibronectin receptor of keratinocytes and α3 that associates with β1 to form the laminin receptor of migrating keratinocytes are contained in type I macroaggregates. By double staining experiments we observed a clear colocalization of β1 integrin with α5 and α3 pointing to the preservation of complete heterodimer receptor complexes during macroaggregate formation (not shown).

Staining of cells and migration tracks with antibodies against hemidesmosomal β4 integrin resulted in a totally different staining pattern with no distinct structures comparable to the type I arrays (Fig. 2b). In contrast we observed a patch-like staining pattern reminding us of the distribution of type II macroaggregates as visualized by EM (Fig. 1c). By immuno-gold labeling we could show that β4 integrin was actually localized to type II macroaggregates (Fig. 2d).

Double immunofluorescence staining (Fig. 3a–c) and double immuno-gold labeling (Fig. 3d) showed no co-localization of β1 and β4 integrin within the migration tracks pointing to both types of macroaggregates as strictly separated compartments presumably originating from the detachment of different types of cell-substrate adhesions.

Quantification of integrin loss
To quantify the loss of β1 and β4 integrins during the formation of macroaggregates, we measured the intensity of fluorescence emission in the migration tracks of β1 and β4 integrin-labeled keratinocytes and calculated the percent of total fluorescence intensity, i.e., the total emission from cell body and migration track. To quantitatively correlate the amount of integrins in the migration track and the migration velocity we classified the keratinocytes according to the length of their migration tracks as slow (track length < 35 μm), intermediate (35 to 80 μm) and fast (>80 μm). After 24 hours of cultivation in the presence of EGF we observed a loss of about 11% (±6%) of the cellular β1 integrin as macroaggregates in migration tracks of fast moving cells and about 4% (±2.6%) in the tracks of slowly moving cells (Fig. 4a, black columns). Over the same period of time the loss of β4 integrin was significantly higher reaching about 18% (±13%) for fast and about 8% (±5%) for slow cells (Fig. 4a, gray columns). Intriguingly, the amount of integrins lost during 1 μm of migration decreased with increasing migration velocity from about 0.15% β1 integrin/μm (Fig. 4b, black columns) and about 0.3% β4 integrin/μm (Fig. 4b, gray columns) for slow cells to 0.08% β1 integrin/μm and 0.15% β4 integrin/μm for fast cells. This points to a correlation between migration velocity and the density of material lost on the substrate.
Cytoskeletal and soluble cytosolic proteins are absent from macroaggregates

Since actin and numerous associated proteins contribute to the formation of focal contacts and are linked with the cytosolic domains of integrins, we determined their distribution at the cell rear, where focal contacts are cleaved, and inside the macroaggregates. As shown before for actin, talin, vinculin and zyxin cytoplasmic proteins were present in retracting fibers to their very tips (Kirfel et al., 2003). However, as soon as the macroaggregates rip off from the cell rear, the labeling was lost and not visible in the macroaggregates. The same applies to cytokeratins, as well as to the cytoplasmic adapter proteins BP230 (Fig. 5) and plectin which usually connect cytokeratins to hemidesmosomal integrins but were absent from macroaggregates. Finally, we used antibodies against chaperones of the Hsp70 family (Bukau and Horwich, 1998), which belong to the

Fig. 1. Transmission (a–c) and scanning EM (d, e) of migrating keratinocytes. Numerous retracting fibers (a, arrowheads) are formed at the cell rear during the process of detachment and characteristic migration tracks are left behind (a). These migration tracks contain two types of macroaggregates: (b, c) Type I macroaggregates are tubular and spherical structures with a diameter of about 50–100 nm that were arranged like “pearls on a string”. These type I macroaggregates seemed to derive from fragmentation of retracting fibers (b, arrowheads). Type II macroaggregates are spherical structures with a diameter of about 30–50 nm that were arranged in clusters distributed over the gaps between type I macroaggregates (c, yellow labeled). Obviously, the type II macroaggregates originate from membrane ripping at the cell rear at the interspaces between adjacent retracting fibers (d, e, arrowheads). Bars: 20 µm (a), 2 µm (b), 500 nm (d, e).
**Fig. 2.** Immunofluorescence microscopy (a, b) and immuno-gold EM (c, d) of β1 (a, c) and β4 (b, d) integrins left behind by migrating keratinocytes. The staining pattern of β1 integrin (a) corresponds to the typical distribution of type I macroaggregates with tubular and spherical structures arranged like “pearls on a string”. β4 Integrin was distributed in form of large patches over the migration track (b). By immuno-gold labeling the localization of β1 integrin to type I macroaggregates became evident (c) whereas β4 integrin specifically localized to type II macroaggregates (d). Bars: 20 μm (a, b), 500 nm (c, d).

**Fig. 3.** Double immunofluorescence analysis (a-c) showed that β1 (green) and β4 (red) integrins are left behind by migrating keratinocytes as constituents of two strictly separated compartments. Immuno-gold labeling (d) showed that type II macroaggregates contain high amounts of β4 integrin (large gold particles) whereas type I macroaggregates and retracting fibers are endowed with β1 integrin (small gold particles). Bars: 20 μm (a-c), 200 nm (d).
most abundant cytosolic proteins, to gain insight into the contribution of soluble cytosolic components during the formation of macroaggregates. As for cytoskeletal proteins, we found high amounts of Hsp70 inside the cell but no detectable signal within macroaggregates (Kirfel et al., 2003).

Macroaggregates are completely membrane-covered compartments

After incubation with the fluorescent lipid dye merocyanine 540 cells and migration tracks were fluorescently labeled. The labeling within the migration tracks showed a pattern that corresponded to type I and type II macroaggregates staining patterns pointing to membrane lipids as constituents of both types of macroaggregates (Fig. 6a). To investigate whether macroaggregates are open membrane fragments or membrane-covered compartments we pre-incubated cells before plating with the membrane-permeable fluorescent dye calcein-AM that becomes hydrolyzed within the cell by esterases rendering it negatively charged and membrane impermeable. Our observations show that calcein remains caged within macroaggregates after their detachment from the cell rear pointing to macroaggregates as completely membrane-covered compartments (Fig. 6b, c). After addition of mild detergents such as digitonin or upon incubation with pore-forming toxins such as streptolysin-O the calcein signal instantly faded away from both, the cell and the macroaggregates.

Discussion

The formation of characteristic migration tracks consisting of integrin-containing macroaggregates has been described recently for fast moving keratinocytes (Kirfel et al., 2003) and might represent a specific strategy of rear detachment necessary for efficient migration to occur. This report shows that keratinocyte migration tracks contain two types of macroaggregates that obviously derive from the detachment of focal contacts and hemidesmosomal structures and can be clearly distinguished due to differences in size, distribution and integrin composition (Table 1). Type I macroaggregates are spherical and tubular structures with a diameter of about 50 – 100 nm which are arranged in the migration track like “pearls on a string” and which have been assumed to derive from fragmentation of retracting fibers, i.e., the tubular membrane extensions at the rear of keratinocytes (Kirfel et al., 2003). These type I macroaggregates contain β1 integrin and different α integrins such as α5 and α6 that are known to be constituents of focal contacts (Zamir et al., 2000). The pattern and size of type I macroaggregates are in agreement with data on the size and distribution of focal contacts on the cell surface (Zamir et al., 2000) implying that they might originate from the dissociation of former focal contacts at the cell rear, preferentially at the tips of retracting fibers. As shown before by using antibodies against the cytosolic as well as the extracellular domain, the lost integrins consist of full-length molecules (Kirfel et al., 2003). It has also

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<td>Size</td>
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<td>Shape</td>
<td>Tubular or spherical</td>
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<td>Structure</td>
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<td>Integrins</td>
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been shown before (Kirfel et al., 2003), however, that type I macroaggregates of keratinocyte migration tracks lack cytosolic proteins of the adhesion complex such as actin, talin, and vinculin that are known to associate with β1 integrin at focal contact sites, or which are most common in the cytosol such as the chaperone Hsp70. Apparently, keratinocytes are able to withdraw all these constituents during macroaggregate formation and, thereby, to minimize their loss during migration. Cleavage of actin-integrin linkages by calpain has been observed and may be a mechanism underlying the withdrawal of actin during membrane ripping by migrating cells (Huttenlocher et al., 1997). It is, however, unknown by which mechanism other cytosolic proteins are retained. The data on type I macroaggregates point to a specific mechanism of focal contact detachment in migrating keratinocytes that depends on the dissociation of all cytosolic adapter proteins from the integrins leaving the whole molecules attached to their ECM ligands.

Type II macroaggregates are spherical structures with a diameter of about 30–50 nm which are left behind in the gaps between adjacent retracting fibers. The new finding in this report is that type II macroaggregates, which lack any of the integrins found in type I macroaggregates (Kirfel et al., 2003), contain considerable amounts of up to 20% of β4 integrins. β4 Integrin, which is expressed primarily on the basal surface of most epithelia including the basal keratinocyte layer of the epidermis, is known as a hemidesmosomal adhesion receptor for basement membrane laminins (Mercurio and Rabinovitz, 2001; Mercurio et al., 2001). Originally hemidesmosomes have been assumed to be non-dynamic structures that are absent from migrating cells including keratinocytes (Carter et al.
1990). However, recent studies on rat epithelial 804G cells showed that during migration β4 integrins progressively assemble and disassemble into cat paw-like arrays on the cell surface (Tsuruta et al., 2003) indicating that hemidesmosomes might be dynamic structures which transiently form during cell migration. Intriguingly, the cat paw-like pattern described by Tsuruta et al. (2003) is in agreement with the pattern we observed for β4-macroaggregates in keratinocyte migration tracks. Obviously keratinocyte migration is also accompanied by the transient formation and subsequent disruption of hemidesmosomes including the loss of β4 integrins at the cell rear. In COS-7 cells serine-phosphorylation of β4 integrins upon stimulation with EGF has been shown to mediate hemidesmosome disassembly by weakening the interaction between the integrins and their cytoplasmic adapters (Rabinovitz et al., 2004). It is as yet unknown whether phosphorylation events might also contribute to the disassembly of hemidesmosomes in keratinocytes and the release of β4 integrins in the form of macroaggregates.

Upon disconnection from the retracting fibers keratinocyte macroaggregates have been shown to remain almost unchanged in size and integrin content for prolonged periods of time, i.e., for at least 24 hours (Kirfel et al., 2003). Obviously, there is no further fragmentation as described for the macroaggregates left behind by mouse fibroblasts (Fuhr et al., 1998) that have been proposed to represent open remnants by Palecek et al. (1998). In contrast, our findings based on the stability of macroaggregates, the caging of calcine and the presence of lipids as shown by mercocyanine staining point to membrane-covered compartments that are impermeable against the diffusion of charged molecules. This release of completely membrane-covered structures as a consequence of rear detachment has not been described before and might represent a new mechanism of focal contact and hemidesmosome disassembly during efficient keratinocyte migration.

**References**


