Integrin cleavage regulates bidirectional signalling in vascular smooth muscle cells

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Introduction

Proliferation and migration of vascular smooth muscle cells (VSMCs) is crucial for vessel development, the initiation and progression of atherosclerosis as well as for the arterial response to injury (1). VSMC motility is triggered by the activation of intracellular signalling cascades, bi-directionally affecting the cytoskeleton (2, 3). Serving as molecular bridges between the extracellular matrix (ECM) and the cytoskeleton, these cell surface receptors mediate signalling in both directions (4, 5). Outside-in signalling is induced by integrin-ligand (e.g., vitronectin) binding, regulating proliferation and survival (4, 6). In contrast, inside-out signalling typically involves the activation of signalling pathways via growth factor receptor activation, subsequently affecting integrin affinity/clustering, which thereby enables rapid changes in cell adhesion and migration (7, 8).

Among the integrin α subunits, some are unique (α3, α5, α6, α7, α8, α9, α11b, αE and αv) in that they are derived from endoproteolytic cleavage of their pro-peptides. Integrin α subunit processing is spatially and temporally regulated during development, conserved among species and does not affect integrin heterodimerisation or membrane expression, indicating a function well beyond biosynthesis (9–12). It typically requires the presence of suitable proprotein convertases (PCs), with furin representing the prototype (13). Furin-dependent αv endoproteolytic cleavage occurs at Lys/Arg-Arg↓ and results in the generation of di-sulphide linked “light” (25 kDa; C-terminal) and “heavy” chains (125 kDa; N-terminal) (14). In vitro, blockade of furin and thus inhibition of αv processing abolished the activation of integrin-associated non-receptor tyrosine kinases, being essential for VSMC adhesion to migration on the αvβ3/5-ligand vitronectin (15).

However, little is known about the consequences of αv subunit processing on inside-out signalling in VSMCs. In K562 leukaemic cells...
uncleavable α6A integrin mutants were resistant to phorbol 12-myristate 13-acetate (PMA) induced inside-out signalling (10). In contrast, Berthet et al. reported that inhibition of αv processing enhanced inside-out mediated haptotaxis in adenocarcinoma cells (16).

Thus, the present study elucidates the impact of furin-dependent αv processing on inside-out / outside-in mediated integrin adaptor protein phosphorylation and dependent signalling cascades, as well as on cytoskeleton/local adhesion dynamics, and adhesion/migration in primary VSMCs.

Materials and methods

Cell culture media, fetal calf serum (FCS), bovine serum albumin (BSA), goat serum and supplements were purchased from Invitrogen (San Diego, CA, USA). The furin-like PC inhibitor decanoyl-RVKR-chloromethylketone (dec-CMK) and cyclic RGD-/RGD-containing hexamers were from Bachem (Bubendorf, Switzerland). Ro32–0432, PD98059, wortmannin, U0124, U0126 and rapamycin were from Calbiochem (La Jolla, CA, USA). IGF-1 was from PreproTech (Rocky Hill, NJ, USA) and PMA was purchased from Sigma Aldrich (St. Louis, MO, USA). The following antibodies were used: anti-αv-integrin [N-terminus, VNR139; Calbiochem; or C-terminus, AB1930; Chemicon (Temecula, CA, USA)], anti-furin (MON-152; Enzo Life Science, Lörrach, Germany), and anti-actin (Sigma Aldrich). Antibodies recognising ERK, phospho-ERK (Thr202/Tyr204), Akt, phospho-Akt (Ser473), phospho-p70S6-kinase (Thr 389), phospho-FAK (Tyr397), phospho-paxillin (Tyr118) and phospho-PKCα were from Cell Signaling (Beverly, MA, USA). Fluorescently labelled phalloidin was from Molecular Probes (Eugene, OR, USA). HRP-conjugated secondary antibodies, fluorescein isothiocyanate (FITC)- and Texas Red (TXR)-conjugated antibodies were from Vector Laboratories (Burlingame, CA, USA). Gelatin and vitronectin were purchased from Sigma Aldrich.

Cell culture

Rat vascular smooth muscle cells (VSMCs) were isolated and cultured as described previously (15). VSMCs (passage 3 – 6) were synchronised by serum-deprivation (0%FCS) overnight. FITC- or TXR-conjugated secondary antibodies were used and nuclei stained with 4'-6-Diamidino-2-phenylindole (DAPI).

Immunoblotting

Immunoblotting was performed as reported, with proteins being resolved on 10% reducing or non-reducing SDS–PAGE (15).

Migration

Chemotaxis experiments were done with a transwell chamber-system using 10%FCS as a chemoattractant (15). Polycarbonate filters with 8 μm pores (Becton Dickinson, Franklin Lakes, NJ, USA) were coated with vitronectin (10 μg/ml). The number of VSMCs per high power field (HPF, 320x magnification) that had migrated to the lower surface of the filters after 4 h under culture conditions was determined microscopically. Four randomly chosen HPFs were counted per well.

Adhesion assay

Microtiter plates (96-wells) were coated with rat vitronectin (10 μg/ml, 16 h, 4°C). Plates were washed with PBS, followed by incubation with PBS containing 1% BSA (60 min at room temperature) to block non-specific binding. VSMCs (30,000/well) were seeded and allowed to adhere for 1 h at 37°C. After washing, attached cells were fixed with 4% paraformaldehyde (pH 7.5), stained with 0.5% toluidine blue and lysed with 1% SDS. Absorbance was measured at 590 nm.

In vitro-2D-wound healing assay

VSMCs (1×10⁵ cells/ml) were seeded, in quadruplicate, on a gelatin (0.2%)-coated 24-well plate and incubated in 10% FCS-containing Dulbecco’s modified eagle medium (DMEM) until confluence. Cells were then synchronised in 0%FCS and treated with dec-CMK or peptides. A 2.0-mm wide ‘wound’ was induced in the centre of the well using a sterile silicone tip. After wounding, cells were incubated with or without 10%FCS, followed by fixation (4% buffered formalin) and staining with fluorescence-phalloidin at indicated times. Measurement of the width of the wound was done using NIH Image J 1.41 software at five different levels per condition.

Immunofluorescence

VSMCs were plated on 10 μg/ml vitronectin-coated chamber-slides (Nunc, Rochester, NY, USA). After adherence, cells were synchronised in serum-free DMEM overnight in the presence or absence of dec-CMK, followed by stimulation with stimuliants, as indicated. Other inhibitors or peptides were given 30 min prior to stimulation, unless indicated otherwise. All experiments were performed in triplicate at the least, with different preparations of VSMCs.

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Statistics

Data are expressed as mean ± standard error of the mean (SEM) of at least three independent experiments. Groups were compared using one-way ANOVA followed by least significance test post-hoc analysis. Statistical significance was designated at a probability value of p<0.05.

Results

The impact of furin on bidirectional integrin signalling in VSMC motility

The effect of furin inhibition and integrin blockade on VSMC motility was assessed by a 2D-wounding assay on a gelatin matrix. Stimulation with serum rapidly led to wound closure (Fig. 1A). This closure was significantly inhibited by pre-incubation of cells with furin-like PC inhibitor dec-CMK (50 μM). RGD- or RGE-peptides (100 nM), cells were left untreated (co.) or were stimulated with 10%FCS. Shown are representative figures 24 h after wounding. B) Quantification of the scratch width after indicated times (*p<0.05 vs. FCS, n=4). C) VSMCs were pre-treated with CMK (50 μM), with or without additional PMA-stimulation (100 nM; 4 h), and were then allowed to migrate towards 10% FCS through vitronectin-coated filters. Data are expressed as percent of FCS-induced migration (mean ± SEM, n=4; **p<0.01 vs. control, #p<0.05 vs. FCS, †p<0.05 vs. PMA). D) VSMCs were treated with PMA (100 nM, 4 h) in the absence or presence of CMK (50 μM). Neither PMA nor CMK affected total protein levels of αv as demonstrated by the 150 kDa non-cleaved (NC) form on non-reducing gels (upper blot). Reducing SDS-PAGE (below) demonstrated that CMK significantly inhibited αv processing to its cleaved (C) 125 kDa form. Protein levels of furin were unaffected by PMA (lower blot). Actin reblotting demonstrated protein loading. (n=3)

Figure 1: Furin regulates VSMC motility via integrin activation.

A) The impact of furin-inhibition on VSMC wound closure was compared to integrin inhibition. Following treatment with the furin-like PC inhibitor dec-CMK (CMK, 50 μM), RGD- or RGE-peptides (100 nM), cells were left untreated (co.) or were stimulated with 10%FCS. Shown are representative figures 24 h after wounding. B) Quantification of the scratch width after indicated times ("p<0.05 vs. FCS, n=4). C) VSMCs were pre-treated with CMK (50 μM), with or without additional PMA-stimulation (100 nM; 4 h), and were then allowed to migrate towards 10% FCS through vitronectin-coated filters. Data are expressed as percent of FCS-induced migration (mean ± SEM, n=4; **p<0.01 vs. control, #p<0.05 vs. FCS, †p<0.05 vs. PMA). D) VSMCs were treated with PMA (100 nM, 4 h) in the absence or presence of CMK (50 μM). Neither PMA nor CMK affected total protein levels of αv as demonstrated by the 150 kDa non-cleaved (NC) form on non-reducing gels (upper blot). Reducing SDS-PAGE (below) demonstrated that CMK significantly inhibited αv processing to its cleaved (C) 125 kDa form. Protein levels of furin were unaffected by PMA (lower blot). Actin reblotting demonstrated protein loading. (n=3)
with αv-blocking RGD-sequence containing cyclic hexapeptides, confirming that RGD-binding integrins are crucial mediators of cell motility. Comparably, furin-inhibition with the furin-like PC inhibitor dec-CMK significantly attenuated wound closure (both *p<0.05 vs. FCS) (Fig. 1A and B).

Transwell migration chambers were used to investigate the requirement of furin for VSMC chemotaxis. Filters were coated with the specific αβ3/5-ligand vitronectin. Cells were pre-treated with dec-CMK and then subjected to PMA-stimulation, with the latter inducing inside-out signalling (4, 17). Furin-blockade significantly inhibited serum-induced VSMC migration through vitronectin (†p<0.05 vs. FCS alone; Fig. 1C). Likewise, dec-CMK reduced migration when inside-out integrin signalling was induced (†p<0.05 vs. PMA-treated cells; Fig. 1C). The inhibitory impact of furin-inhibition, however, was less pronounced in PMA-stimulated cells.

We further investigated the impact of PMA on the expression of αv and its convertase furin in VSMCs. PMA treatment did neither affect total αv nor furin protein levels (Fig. 1D). In line with our previous studies (15), furin-inhibition with dec-CMK inhibited αv processing from its 150 kDa precursor to the mature 125 kDa form in PMA-stimulated cells as well.

Integrin inside-out signalling is not affected by furin-dependent αv processing

PMA has been demonstrated to activate several signalling pathways including PKC and MEK/ERK (4, 18). In accordance, stimulation of VSMCs with PMA for 30 min increased phosphorylation...
levels of PKCα/β1 isoforms and ERK1/2 (Fig. 2A). Phosphorylation of ERK1/2 was effectively suppressed by PKC attenuation due to long-term (24 h) PMA treatment (19) (data not shown), as well as by pre-treatment with the PKC-inhibitor Ro32–0432 (Fig. 2B). Thus, PMA-dependent ERK activation is a consequence of PKC. A detailed time-course analysis (up to 4 h) revealed significantly prolonged phosphorylation of ERK1/2 and p70s6-kinase by PKC-activation, whereas Akt was only briefly phosphorylated (maximum at 10 min) (Fig. 2C, left panel). Activation of p70s6-kinase was blocked by the MEK1-inhibitor PD98059 (data not shown). Thus, PKC seems to be proximal in the signalling cascade, activating ERK1/2, which further regulates p70s6-kinase. Interestingly, inhibition of integrin cleavage via the inhibition of furin with dec-CMK had no impact on PMA-stimulated inside-out signalling pathways (Fig. 2C, right panel).

To further investigate whether PMA-induced signalling pathways mediate αv-dependent VSMC functions, adhesion and migration assays were performed on the αvβ3/5-ligand vitronectin. Outside-in mediated VSMC adhesion was significantly inhibited by pre-incubation of cells with αv-blocking RGD peptides (Fig. 2D). Induction of integrin inside-out signalling by PMA significantly increased VSMC adhesion, which was inhibited by RGD-, but not by control RGE peptides (Fig. 2E). Furthermore, inhibition of cells with the specific pharmacological inhibitors of PKC (Ro32–0432), MEK1 (PD98059) or mTOR (rapamycin) significantly inhibited inside-out mediated VSMC adhesion and migration (Fig. 2E and F). Thus, PMA-mediated inside-out signalling is sufficient to induce integrin-mediated VSMC functions and depends on PKC/ERK/p70s6-kinase activation, whereas Akt seemed to be of minor relevance (Fig. 2C).

Interestingly, previous reports have suggested that activation of p70s6-kinase is essentially downstream of Akt pathways (20). However, more recent studies support the notion that other signalling pathways may contribute (21, 22). Therefore, we compared the regulation of p70s6-kinase by ERK1/2 with Akt signalling in VSMCs. Cells were stimulated with PMA which has been shown to primarily activate ERK1/2 but not Akt, or with insulin-like growth factor-1 (IGF-1) which mainly induces Akt but not ERK1/2 in VSMCs (19, 23). Both PMA and IGF-1 led to p70s6-kinase phosphorylation, which was abolished by the mTOR-inhibitor rapamycin (Fig. 3A and B). However, PMA-mediated p70s6-kinase activation was inhibited by the MEK1/2-inhibitor U0126, but was unaffected by its control U0124 or the phosphatidylinositol 3-OH-kinase (PI3K)-inhibitor wortmannin. In contrast, IGF-1-mediated p70s6-kinase phosphorylation was strongly inhibited by wortmannin, but not by MEK-inhibition. Thus, IGF-1-induced p70s6-kinase is in particular PI3K-dependent, whereas inside-out PMA-mediated p70s6-kinase activation relies on PKC-dependent ERK-pathways.

**Figure 3: Diverse activation of p70s6-kinase in VSMCs.** A) VSMCs were left resting (co.), or were treated with PMA (100 nM, 30 min), or U0126 or its control U0124 (both 2 μM), or wortmannin (wortm., 50 nM). Immunoblotting was done using antibodies as indicated. (n=3)

**Furin-dependent αv cleavage diversely affects the activation of focal adhesion proteins in integrin bidirectional signalling**

Imaging and immunoblotting experiments were used to investigate the effect of furin-dependent αv cleavage on the activation of integrin adapter proteins and on focal adhesion formation. First, VSMCs were pre-treated with dec-CMK and then either subjected to adhesion to the αvβ3/5-ligand vitronectin – to solely induce integrin-mediated outside-in signalling – or cells were stimulated with PMA to induce PKC-mediated integrin inside-out signalling. Adhesion to vitronectin strongly enhanced phosphorylation of FAK and paxillin, which was abolished by furin inhibition (Fig. 4A, left panel). In contrast, inhibition of αv processing did not affect inside-out mediated phosphorylation of FAK and paxillin (Fig. 4A, right panel). This underlines that uncleaved αv maintains responsiveness for inside-out triggered signalling events. However, PMA-induced activation of focal adhesion proteins was significantly lessened by PKC- or MEK-inhibition, suggesting that FAK and paxillin activation depends on these pathways in inside-out signalling (Fig. 4A, right panel). To further demonstrate that inside-out dependent integrin ligation mediates FAK and paxillin phosphorylation, vitronectin adherent VSMCs were incubated with αv-blocking RGD peptides. These impaired phosphorylation...
of focal adhesion proteins, whereas control RGE peptides had no effect (Fig. 4B).

We then elucidated the impact of furin-dependent $\alpha_v$ processing on inside-out mediated focal adhesion formation and cytoskeleton remodeling, using double-labeling immunofluorescence studies with VSMCs on vitronectin coated chamber slides. Whereas PMA-stimulated inside-out signalling led to the recruitment of $\alpha_v$ in close proximity with actin filaments at focal adhesion sites (Fig. 4C), inhibition of furin and thus inhibition of $\alpha_v$ processing partially inhibited it (Fig. 4D). Hence, detailed additional analyses investigating alterations in cytoskeleton dynamics, actin filament content, and phosphorylation of integrin adaptor proteins were performed (Fig. 5A-1). PMA treatment increased F-actin content, polymerisation and rearrangement to stress fibers (Fig. 5D). This was accompanied by an increase in phosphorylation of paxillin at focal adhesion sites (Fig. 5E and F). In contrast, inhibition of furin-dependent integrin processing by dec-CMK prevented stress fiber formation and increases in actin content, with actin remaining at the surrounding external boundaries of the cells (Fig. 5G). Coming along with this, phospho-paxillin localisation to focal adhesions was lost (Fig. 5H and F). Thus, furin-dependent $\alpha_v$ cleavage affects integrin inside-out mediated remodelling of focal adhesions and the cytoskeleton, but not inside-out dependent signalling pathways.

Figure 4: Bidirectional integrin signalling is diversely affected by furin inhibition. A) VSMCs were pre-treated with CMK (50 μM), and lysed (-VN), or were seeded on vitronectin (+VN)-coated (10 μg/ml) plates for 30 min (left panel). Cells were also left resting (co.) or were treated with inhibitors [dec-CMK (CMK, 50 μM); PD98059 (PD, 30 μM); Ro32–0432 (Ro, 5 μM)], followed by stimulation with PMA (100 nM, 30 min) (right panel). Immunoblotting was done with antibodies against phospho-FAK, phospho-paxillin, and actin. (n=3) B) Vitronectin adherent VSMCs were treated with RGD- or RGE peptides for 1 h, followed by either stimulation with PMA (100 nM, 30 min) or with buffer (-). Cells were lysed and extracted proteins were subjected to standard immunoblotting using antibodies as indicated. (n=3) C-D) Vitronectin-adherent VSMCs were left untreated (C) or pretreated (D) with dec-CMK (50 μM). Association of $\alpha_v$ (green) with F-actin (red) at focal adhesion sites was investigated following PKC-activation with PMA (100 nM, 4 h) using double-labelling immunofluorescence. Whereas PMA-stimulation led to the targeting of $\alpha_v$ (arrows) to focal adhesion sites, this was partly lost by the inhibition of furin-dependent integrin processing with dec-CMK. Nuclei are stained with DAPI (blue). (n=4)
Discussion

The present study demonstrates that furin-dependent αv cleavage profoundly affects integrin bidirectional signalling crucially impacting on VSMC migration. Inhibition of αv processing inhibited integrin outside-in (vitronectin)-induced tyrosine phosphorylation of FAK and paxillin, as well as chemotaxis and wound closure. In contrast, when integrin inside-out signalling was induced, activation of integrin-adaptor proteins was maintained, but cytoskeleton remodeling and focal adhesion formation were hampered by the status of αv cleavage. Hence, in VSMCs the integrin convertase furin is central in the rapid adaptive changes and regulations governing ECM – cytoskeleton communication in either direction.

The proprotein convertase (PC) furin is typically expressed at low levels under physiological conditions but up-regulated in diseases such as cancer and atherosclerosis (13, 24). Based on their potential substrates, PCs have been suggested as pharmacological targets, and inhibition of furin has been shown to attenuate cancer cell migration/metastasis, as well as neointima formation (13, 25). Although the general PC-cleavage motif indicates multiple substrates, substrate activation in vivo is controlled by unique enzymes/substrate expression patterns, and sequence/biophysical requirements in substrate pro-peptides (13). Furin and αv are coordinately up-regulated following balloon injury in rodents and are co-expressed in human atheroma (15, 24). In VSMCs, furin-dependent αv cleavage occurs in the trans-Golgi network, and follows α/β subunits assembly in the endoplasmic reticulum (12, 24). Prevention from cleavage has been shown to neither affect heterodimerisation nor membrane expression of integrins (9, 10, 12). Furin-like PC-inhibition either with pharmacological inhibitors or with specific antisense oligonucleotides inhibited αv-dependent VSMC adhesion to vitronectin, but not to collagen I, which depends on α2 integrins (15). Interestingly, Berthet et al. reported that uncleaved and cleaved α-integrins are equally effective in ligand binding, and that uncleaved αvβ5 maintained the capability to mediate adhesion when inside-out signalling was induced in carcinoma cells (9). In their experiments, cells over-expressing the furin-inhibitor α1-PDX (α1-antitrypsin Portland) were found to display readily established focal adhesion sites and increased stress fiber formation. Over-expression of this serpin variant resulted in enhanced inside-out migration while αv processing was inhibited (9). However, additional genes might have been affected by this inhibitor. Accordingly, it was recently demonstrated that α1-PDX increases MMP-9 mediated cell motility (26).

Using the specific pharmacological furin-like PC inhibitor dec-CMK (27), our study demonstrates that inhibition of αv cleavage does not affect inside-out mediated phosphorylation of FAK and paxillin, whereas ligand-binding induced (outside-in) phosphorylation of these integrin-adaptor proteins was significantly impaired. Inhibition of integrin cleavage, however, hampered inside-out mediated recruitment of non-receptor tyrosine kinases to focal adhesion sites and cytoskeleton rearrangement, thereby lessening VSMC motility. Elucidating the relevant downstream signalling events responsible for the activation of integrin-adaptor proteins in VSMCs, we demonstrate that inside-out integrin activation depends on phosphorylation of PKC, and subsequent phosphorylation of ERK, as found earlier in other cell types (28). ERK phosphorylation upon PKC activation displayed a prolonged pattern, while phosphorylation of the PI3K downstream signalling molecule Akt (protein kinase B) was brief. Concomitant with sustained ERK phosphorylation, our study shows prolonged phosphorylation of p70s6-kinase, which is crucial for cell cycle progression, growth and motility of VSMCs in vascular remodelling (29). MEK-inhibition abolished inside-out induced phosphorylation of p70s6-kinase. In contrast, inhibition of Akt did not significantly affect phosphorylation of p70s6-kinase. Thus, while Akt was pre-
viously acknowledged as main upstream regulator of p70s6-kinase (20), our study suggests rather ERK than Akt being essential for PKC-mediated p70s6-kinase phosphorylation in inside-out signalling. In fact, distinct pathways leading to activation of p70s6-kinase have been observed, including PKC/c-Raf/MEK/ERK signalling (21). In line with this, PI3K but not MEK inhibition blunted p70s6-kinase phosphorylation when Akt was induced by IGF-1 (19, 23). In either way, p70s6-kinase activation was inhibited by rapamycin, indicating that despite multiple signalling pathways regulate p70s6-kinase in VSMCs, they converge at mTOR.

Our data further demonstrate that inhibition of PKC and ERK, the latter localising at focal adhesion sites upon PKC stimulation (30), abolished inside-out mediated phosphorylation of FAK and paxillin. Comparably to the impact of inhibition of PKC and ERK resulted in attenuated VSMC adhesion and migration. Thus, PKC → ERK-dependent phosphorylation of non-receptor tyrosine kinases is essential in αvβ3-mediated integrin-dependent VSMC motility.

In conclusion, our study suggests furin as a gatekeeper in ECM – cytoskeleton communication essential for bidirectional signalling, cytoskeleton remodeling and focal adhesion formation. This is mediated via furin’s unique capacity as integrin convertase. Blockade of integrin processing via the inhibition of furin may provide a novel approach to modulate integrin signalling and motility in VSMCs, which are integral components in atherosclerosis and restenosis.

References