

Integrin cleavage regulates bidirectional signalling in vascular smooth muscle cells

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Summary

Integrins link the cytoskeleton to the extracellular matrix, providing outside-in/inside-out signalling essential for vascular smooth muscle cell (VSMC) migration in atherosclerosis. The integrin αv subunit is synthesised from its precursor via furin-dependent endoproteolytic cleavage. Furin is a proprotein convertase (PC) highly expressed in VSMCs and in human atherosclerotic lesions. Inhibition of αv processing inhibits binding to vitronectin and migration. However, the precise role of furin-dependent αv cleavage on integrin bidirectional signalling and subsequent VSMC functions is unknown. Our present study demonstrates that the furin-like PC inhibitor decanoyl-RVKR-chloromethylketone (dec-CMK) inhibited αv cleavage. This reduced vitronectin-induced (outside-in) focal adhesion kinase (FAK)- and paxillin-phosphorylation, and VSMC motility. Inside-out-stimulated, integrin-mediated VSMC adhesion/migration relied on integrin-adaptor protein

activation following protein kinase C (PKC) and ERK1/2 phosphorylation. In contrast to outside-in signalling, PKC-dependent phosphorylation of FAK and paxillin was unaffected by the status of integrin cleavage. Still, cytoskeleton and focal adhesion site rearrangements were modulated by the inhibition of furin-dependent integrin cleavage, thereby lessening inside-out dependent migration. Hence, we find that integrin bidirectional signalling is critically controlled by furin. Furin-dependent integrin processing modulates rapid adaptive integrin/cytoskeleton changes, essential to VSMC motility, which represents a crucial component in atherosclerosis and restenosis.

Keywords

Furin, proprotein convertase, integrin, cleavage, 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 atherogenesis as well as for the arterial response to injury (1). VSMC motility is triggered by the activation of intracellular signalling cascades, subsequently involving cytoskeleton reorganisation and focal contact remodeling, in which activation of α/β integrins is an essential part (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 ($\alpha 3$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 8$, $\alpha 9$, αIIb , 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 $\alpha v\beta 3/5$ -ligand vitronectin (15).

However, little is known about the consequences of α subunit processing on *inside-out* signalling in VSMCs. In K562 leukaemic cells

uncleavable $\alpha 6 A$ 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/focal 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- /RGE-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 (Thr³⁸⁹), phospho-FAK (Tyr³⁹⁷), phospho-paxillin (Tyr¹¹⁸) and phospho-PKC α / β II (Thr^{638/641}) 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 in the presence or absence of dec-CMK, followed by treatment with stimulants, 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.

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 μ m pores (Becton Dickinson, Franklin Lakes, NJ, USA) were coated with vitronectin (10 μ g/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 μ g/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 (1×10^5 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 μ g/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 as indicated. Cells were fixed with 4% buffered formalin and non-specific binding blocked with 10% goat serum. Following washing with PBS/0.01% Triton-X100, incubation with primary antibodies (1:50) was done overnight. FITC- or TXR-conjugated secondary antibodies were used and nuclei stained with 4'-6-Diamidino-2-phenylindole (DAPI).

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

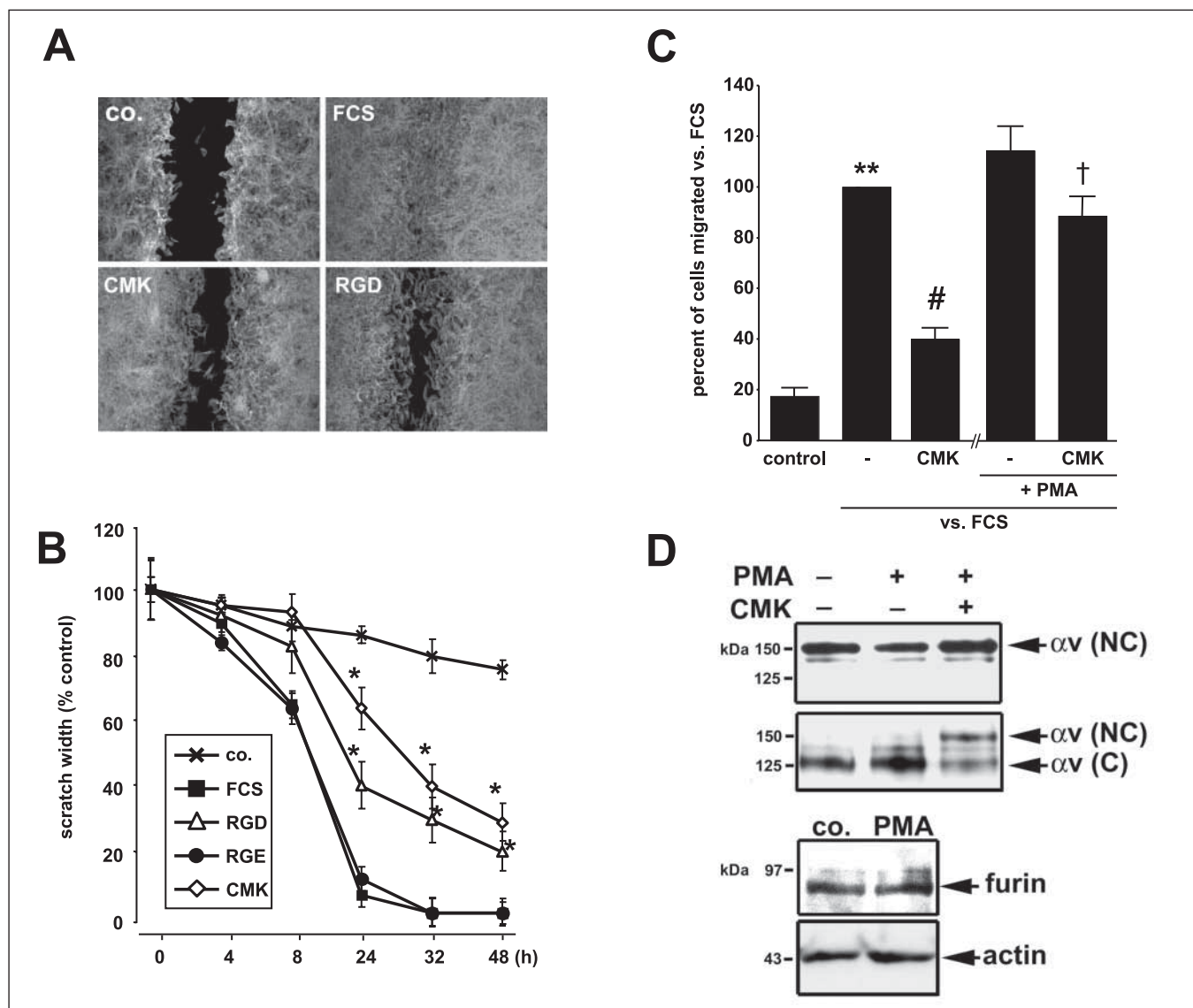


Figure 1: Furin regulates VSMC motility via integrin activation.

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 $\alpha v \beta 3/5$ -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 ($\dagger p < 0.05$ 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

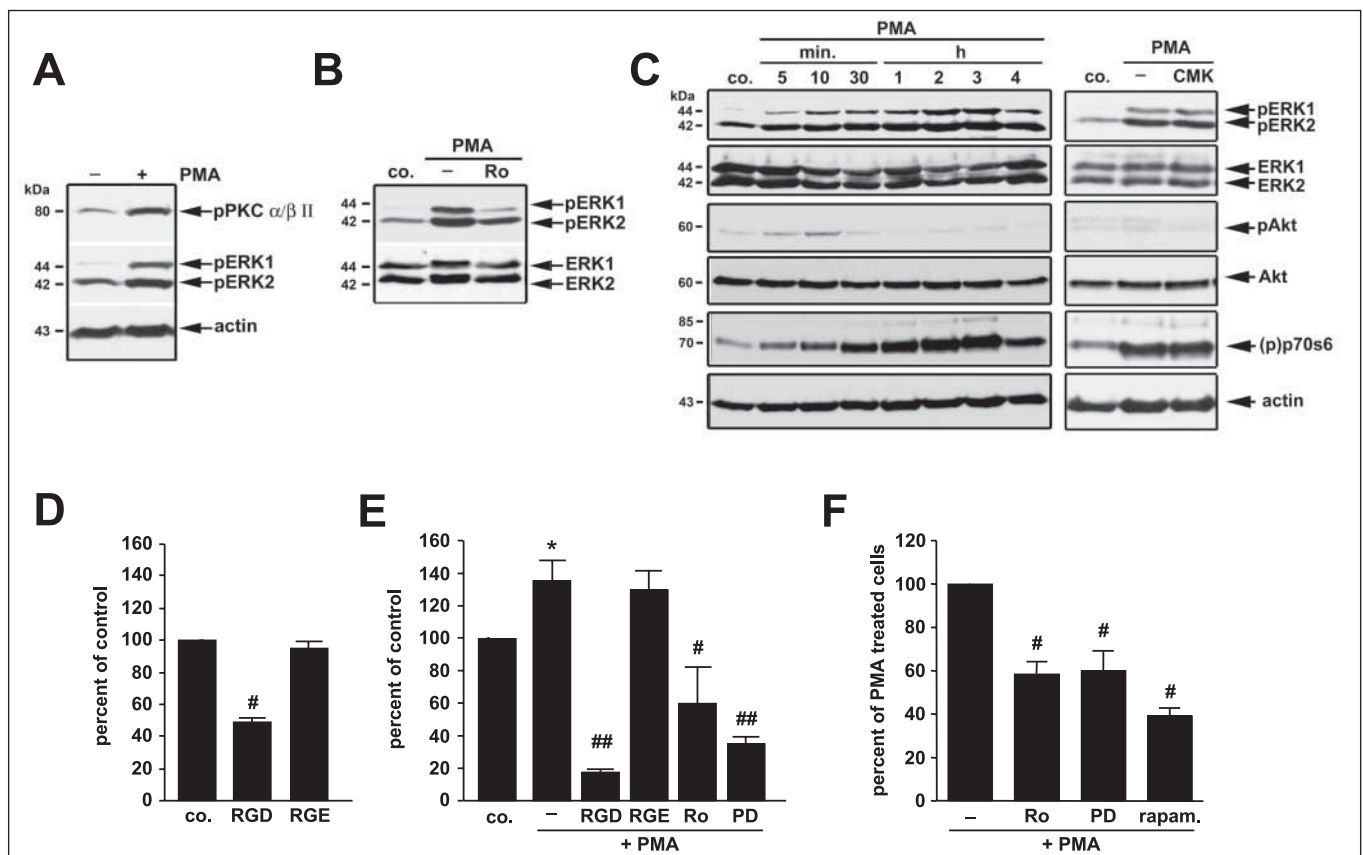


Figure 2: Inside-out signalling cascades and αv -dependent VSMC motility. A) VSMCs were left resting (-) or were stimulated with PMA (+) (100 nM, 30 min) and proteins were immunoblotted as indicated. (n=3). B) VSMCs were left resting (co.) or were stimulated with PMA (100 nM) for 30 min in the absence (-) or presence of the PKC-inhibitor Ro32-0432 (Ro, 5 μ M) (n=3). C) Cells were left resting (co.) or were stimulated with PMA (100 nM) for indicated times. Proteins were subjected to immunoblotting using antibodies as indicated (left panel). Furthermore, cells were pre-treated with dec-CMK (50 μ M), followed by PMA (100 nM, 30 min) stimulation (right panel). (n=3). D) VSMCs were pre-treated without (co.) or with RGD-, and RGE peptides (100 nM, each respectively), for 30 min and were allowed to adhere on vitronectin for 1 h. Adhesion is expressed as percent of untreated cells (mean

\pm SEM; n=3; $\#p < 0.05$ vs. control (co.)). E) Cells were left untreated (co.) or were treated with PMA (100 nM, 30 min), with or without additional treatment with the pharmacological inhibitors Ro32-0432 (Ro, 5 μ M) and PD98059 (PD 30 μ M), or RGD- and RGE peptides (100 nM). Cells were allowed to adhere for 1 h on vitronectin and data are expressed as percent of untreated controls (co.) (mean \pm SEM; n=3; $*p < 0.05$ vs. untreated cells; $\#p < 0.05$ vs. PMA; $\##p < 0.01$ vs. PMA). F) VSMCs were pre-treated with inhibitors (Ro32-0432 (5 μ M); PD98059 (30 μ M); rapamycin (rapam., 10 ng/ml)), followed by PMA-stimulation (100 nM), and then allowed to migrate towards 10% FCS through vitronectin-coated filters. Data are expressed as percent of PMA-treatment (mean \pm SEM, n=4; $\#p < 0.05$ vs. PMA).

levels of PKC α / β II 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 α v-dependent VSMC functions, adhesion and migration assays were performed on the α v β 3/5-ligand vitronectin. *Outside-in* mediated VSMC adhesion was significantly inhibited by pre-incubation of cells with α v-blocking RGD peptides (Fig. 2D). Induction of integrin *inside-out* signalling by PMA signifi-

cantly 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.

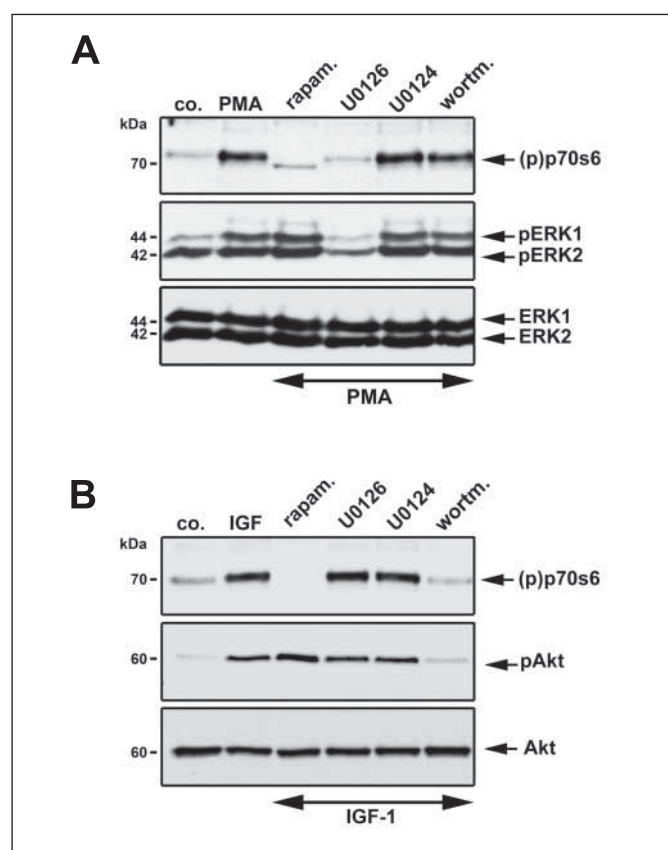


Figure 3: Diverse activation of p70s6-kinase in VSMCs. A) VSMCs were left resting (co.), or were treated with PMA (100 nM, 30 min), or (B) IGF-1 (50 ng/ml, 30 min), in the absence or presence of rapamycin (10 ng/ml), U0126 or its control U0124 (both 2 μ M), or wortmannin (wortm., 50 nM). Immunoblotting was done using antibodies as indicated. (n=3)

Furin-dependent α v 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 α v 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 α v β 3/5-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 α v processing did not affect *inside-out* mediated phosphorylation of FAK and paxillin (Fig. 4A, right panel). This underlines that uncleaved α v 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 α v-blocking RGD peptides. These impaired phosphorylation

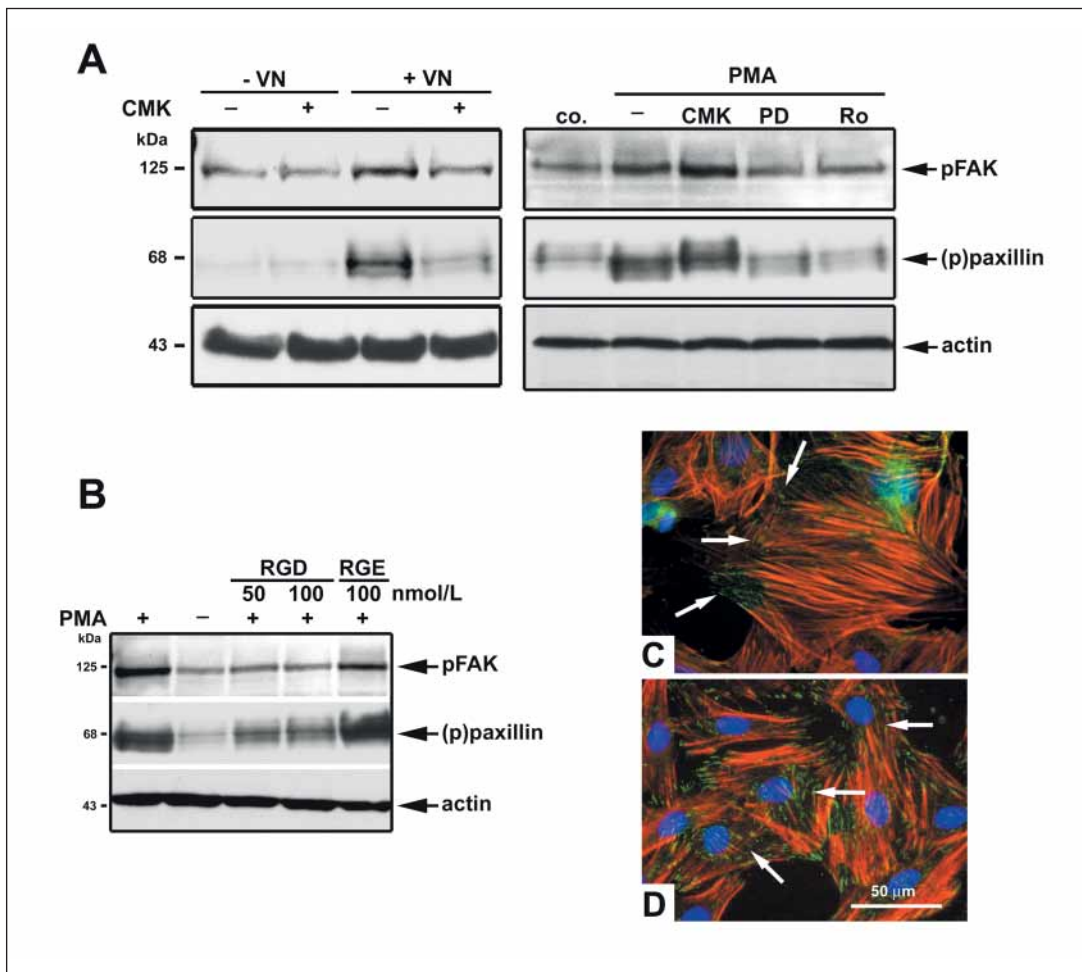


Figure 4: Bidirectional integrin signalling is diversely affected by furin inhibition. A) VSMCs were pre-treated with CMK (50 μ M), and lysed (-VN), or were seeded on vitronectin (+VN)-coated (10 μ g/ml) plates for 30 min (left panel). Cells were also left resting (co.) or were treated with inhibitors [dec-CMK (CMK, 50 μ M); PD98059 (PD, 30 μ M); Ro32-0432 (Ro, 5 μ M)], 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 μ M). Association of α 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 α 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)

of focal adhesion proteins, whereas control RGE peptides had no effect (Fig. 4B).

We then elucidated the impact of furin-dependent α 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 α v in close proximity with actin filaments at focal adhesion sites (Fig. 4C), inhibition of furin and thus inhibition of α 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-I). 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 α v cleavage affects integrin *inside-out* mediated remodelling of focal adhesions and the cytoskeleton, but not *inside-out* dependent signalling pathways.

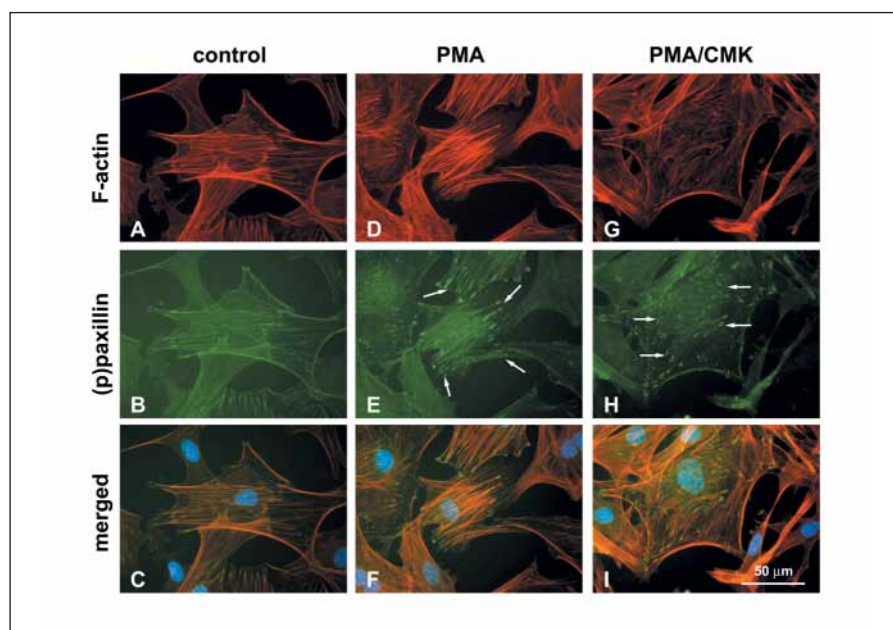


Figure 5: The impact of furin-inhibition and thus inhibition of αv integrin processing on actin cytoskeleton dynamics and localisation of phospho-adaptor proteins was investigated. F-actin was visualised using fluorescently labeled phalloidin (red), and an antibody against phospho-paxillin (green, arrows) was used to investigate its trans-localisation to focal adhesions. Vitronectin (10 $\mu\text{g}/\text{ml}$) adherent cells were left untreated (A, B, C), were treated with PMA (100 nM) (D, E, F), or were pre-treated with dec-CMK (50 μM), followed by PMA stimulation (G, H, I). Nuclei were 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 $\alpha 2$ integrins (15). Interestingly, Berthet et al. reported

that uncleaved and cleaved α -integrins are equally effective in ligand binding, and that uncleaved $\alpha v\beta 5$ 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 $\alpha 1$ -PDX ($\alpha 1$ -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 $\alpha 1$ -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 αv processing or integrin blockade, 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 αv *inside-out* 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

- Doran AC, Meller N, McNamara CA. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arterioscler Thromb Vasc Biol* 2008; 28: 812–819.
- Gerthoffer WT. Mechanisms of vascular smooth muscle cell migration. *Circ Res* 2007; 100: 607–621.
- Haas TA. Discrete functional motifs reside within the cytoplasmic tail of alpha V integrin subunit. *Thromb Haemost* 2008; 99: 96–107.
- Coppolino MG, Dedhar S. Bi-directional signal transduction by integrin receptors. *Int J Biochem Cell Biol* 2000; 32: 171–188.
- Petrich BG. Talin-dependent integrin signalling in vivo. *Thromb Haemost* 2009; 101: 1020–1024.
- Bilato C, Curto KA, Monticone RE, et al. The inhibition of vascular smooth muscle cell migration by peptide and antibody antagonists of the alpha v beta 3 integrin complex is reversed by activated calcium/calmodulin-dependent protein kinase II. *J Clin Invest* 1997; 100: 693–704.
- Sekimoto H, Eipper-Mains J, Pond-Tor S, et al. (alpha)v(beta)3 integrins and Pyk2 mediate insulin-like growth factor I activation of Src and mitogen-activated protein kinase in 3T3-L1 cells. *Mol Endocrinol* 2005; 19: 1859–1867.
- Stawowy P, Margeta C, Blaschke F, et al. Protein kinase C epsilon mediates angiotensin II-induced activation of beta1-integrins in cardiac fibroblasts. *Cardiovasc Res* 2005; 67: 50–59.
- Berthet V, Rigot V, Champion S, et al. Role of endoproteolytic processing in the adhesive and signalling functions of alpha v beta 5 integrin. *J Biol Chem* 2000; 275: 33308–33313.
- Delwel GO, Hogervorst F, Sonnenberg A. Cleavage of the alpha 6A subunit is essential for activation of the alpha 6A beta 1 integrin by phorbol 12-myristate 13-acetate. *J Biol Chem* 1996; 271: 7293–7296.
- Demetriou MC, Stylianou P, Andreou M, et al. Spatially and temporally regulated alpha6 integrin cleavage during *Xenopus laevis* development. *Biochem Biophys Res Commun* 2008; 366: 779–785.
- Rigot V, Andre F, Lehmann M, et al. Biogenesis of alpha 6 beta 4 integrin in a human colonic adenocarcinoma cell line involvement of calnexin. *Eur J Biochem* 1999; 261: 659–666.
- Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol* 2002; 3: 753–766.
- Lissitzky JC, Luis J, Munzer JS, et al. Endoproteolytic processing of integrin pro-alpha subunits involves the redundant function of furin and proprotein convertase (PC) 5A, but not paired basic amino acid converting enzyme (PACE) 4, PC5B or PC7. *Biochem J* 2000; 346: 133–138.
- Stawowy P, Kallisch H, Veinot JP, et al. Endoproteolytic activation of alpha(v) integrin by proprotein convertase PC5 is required for vascular smooth muscle cell adhesion to vitronectin and integrin-dependent signalling. *Circulation* 2004; 109: 770–776.
- Berthet V, Rigot V, Nejjari M, et al. The endoproteolytic processing of alphavbeta5 integrin is involved in cytoskeleton remodelling and cell migration. *FEBS Lett* 2004; 557: 159–163.
- Disatnik MH, Rando TA. Integrin-mediated muscle cell spreading. The role of protein kinase c in outside-in and inside-out signalling and evidence of integrin cross-talk. *J Biol Chem* 1999; 274: 32486–32492.
- Lee MS, Kim YB, Lee SY, et al. Integrin signalling and cell spreading mediated by phorbol 12-myristate 13-acetate treatment. *J Cell Biochem* 2006; 99: 88–95.
- Yano K, Bauchat JR, Liimatta MB, et al. Down-regulation of protein kinase C inhibits insulin-like growth factor I-induced vascular smooth muscle cell proliferation, migration, and gene expression. *Endocrinology* 1999; 140: 4622–4632.
- Ballou LM, Luther H, Thomas G. MAP2 kinase and 70K S6 kinase lie on distinct signalling pathways. *Nature* 1991; 349: 348–350.
- Iijima Y, Laser M, Shiraishi H, et al. c-Raf/MEK/ERK pathway controls protein kinase C-mediated p70S6K activation in adult cardiac muscle cells. *J Biol Chem* 2002; 277: 23065–23075.
- Ma L, Chen Z, Erdjument-Bromage H, et al. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell* 2005; 121: 179–193.
- Stawowy P, Kallisch H, Kilimnik A, et al. Proprotein convertases regulate insulin-like growth factor I-induced membrane-type 1 matrix metalloproteinase in VSMCs via endoproteolytic activation of the insulin-like growth factor-1 receptor. *Biochem Biophys Res Commun* 2004; 321: 531–538.
- Stawowy P, Graf K, Goetze S, et al. Coordinated regulation and colocalization of alphav integrin and its activating enzyme proprotein convertase PC5 in vivo. *Histochem Cell Biol* 2003; 119: 239–245.
- Sluijter JP, Verloop RE, Pulskens WP, et al. Involvement of furin-like proprotein convertases in the arterial response to injury. *Cardiovasc Res* 2005; 68: 136–143.
- Lapierre M, Siegfried G, Scamuffa N, et al. Opposing function of the proprotein convertases furin and PACE4 on breast cancer cells' malignant phenotypes: role of tissue inhibitors of metalloproteinase-1. *Cancer Res* 2007; 67: 9030–9034.
- Hallenberger S, Bosch V, Angliker H, et al. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. *Nature* 1992; 360: 358–361.
- Rigot V, Lehmann M, Andre F, et al. Integrin ligation and PKC activation are required for migration of colon carcinoma cells. *J Cell Sci* 1998; 111: 3119–3127.
- Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat Med* 2002; 8: 1249–1256.
- Besson A, Davy A, Robbins SM, et al. Differential activation of ERKs to focal adhesions by PKC epsilon is required for PMA-induced adhesion and migration of human glioma cells. *Oncogene* 2001; 20: 7398–7407.