

p190B RhoGAP regulates endothelial-cell-associated proteolysis through MT1-MMP and MMP2

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Summary

The two isoforms of p190 RhoGAP (p190A and p190B) are important regulators of RhoGTPase activity in mammalian cells. Both proteins are ubiquitously expressed, are involved in the same signalling pathways and interact with the same identified binding partners. In search of isoform functional specificity, we knocked down the expression of each p190 protein using siRNA and examined the resulting phenotypic changes in human umbilical vein endothelial cells (HUVECs). We provide evidence that p190B plays a crucial role in the regulation of MT1-MMP expression and cell-surface presentation, as well as subsequent MMP2 activation. p190B is involved in both local extracellular matrix degradation at podosomes and endothelial cell assembly into tube-like structures in Matrigel. In addition,

whereas p190B knockdown does not affect podosome formation, p190A knockdown increases the number of cells showing podosome structures in HUVECs. We conclude that the two p190 RhoGAP isoforms play distinct roles in endothelial cells. In addition, our data reveal an unsuspected role for p190B in the expression of the two collaborative proteases MT1-MMP and MMP2, thereby affecting matrix remodelling and angiogenesis.

Supplementary material available online at
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Key words: p190 RhoGAP, Metalloproteinase, Podosomes

Introduction

Cell migration and invasion play key roles in a plethora of biological events, including embryonic development, immunity, angiogenesis and tumour metastasis. Migration and invasion are typically regulated by actin dynamics, cell-cell and cell-substrate adhesion, and extracellular matrix (ECM) remodelling. In various models, actin-based processes have been shown to be controlled by small G-protein members of the Rho family, a subclass of the Ras superfamily. Rho GTPases cycle between an inactive GDP-bound and an active GTP-bound form. In their active conformation, GTPases are able to interact with and activate their specific effectors. In cells, GTPase activity is tightly regulated by the coordinated action of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs catalyse the exchange of GDP for GTP and hence activate the GTPases, whereas GAPs enhance the intrinsic GTPase activity, returning the GTPases to their basal GDP-bound state. p190 RhoGAP (p190) is an important regulator of RhoGTPase activity in mammalian cells. p190 exists as two related proteins, namely p190A and p190B, encoded in humans by the *GRLF1* and *ARHGAP5* genes, respectively. Both proteins share 51% identity and display similar domain organisation, including an N-terminal GTP-binding segment followed by four FF domains (protein-protein interacting domains harbouring two strictly conserved phenylalanine residues), a central domain and the C-terminal RhoGAP domain. Both proteins are ubiquitously expressed in adults. In mouse embryos, both isoforms are highly expressed in the brain, in which they play both overlapping as well as specific roles. Mice lacking functional p190A exhibit several neuronal defects, reflecting a role for p190A in axon

guidance and fasciculation (Brouns et al., 2001), whereas mice lacking p190B display axonal-tract deficits and neuronal-differentiation defects, pointing to a role for p190B in normal brain development (Matheson et al., 2006). In addition, p190B-deficient mice are 30% reduced in size because of a defect in insulin and IGF1 signalling impacting on adipogenesis functions (Sordella et al., 2002; Sordella et al., 2003). In cells, p190 proteins are recruited to sites of integrin clustering, where they couple signals from the ECM with the actin cytoskeleton through RhoGTPases. Both p190A and p190B exhibit their catalytic activity preferentially towards RhoA. Nevertheless, several phospholipids have been shown to switch this specificity towards Rac (Ligeti et al., 2004). p190A was first described as a tyrosine-phosphorylated protein in a complex with p120 RasGAP in serum-stimulated cells (Ellis et al., 1990; Settleman et al., 1992) and was then identified as a substrate of the Src kinase (Roof et al., 1998). In contrast to p190A, p190B is not abundantly phosphorylated in Src-transformed Rat-1 fibroblasts (Matheson et al., 2006). So far, no difference between the two isoforms has been found at the molecular level. Both isoforms are involved in the same cellular pathways and both interact with p120 RasGAP, Rnd3 and TFII-I (GTF2I) (Burbelo et al., 1995; Jiang et al., 2005; Wennerberg et al., 2003). However, recent studies suggested that p190 RhoGAPs might play more-complex roles than previously thought, and are not solely limited to the regulation of RhoA activity (Matheson et al., 2006).

In addition to actin dynamics, ECM remodelling is also crucial for cell migration and invasion. Among the different proteolytic systems, the matrix metalloproteinases (MMPs) strongly contribute to the cleavage of ECM proteins during invasive processes such as

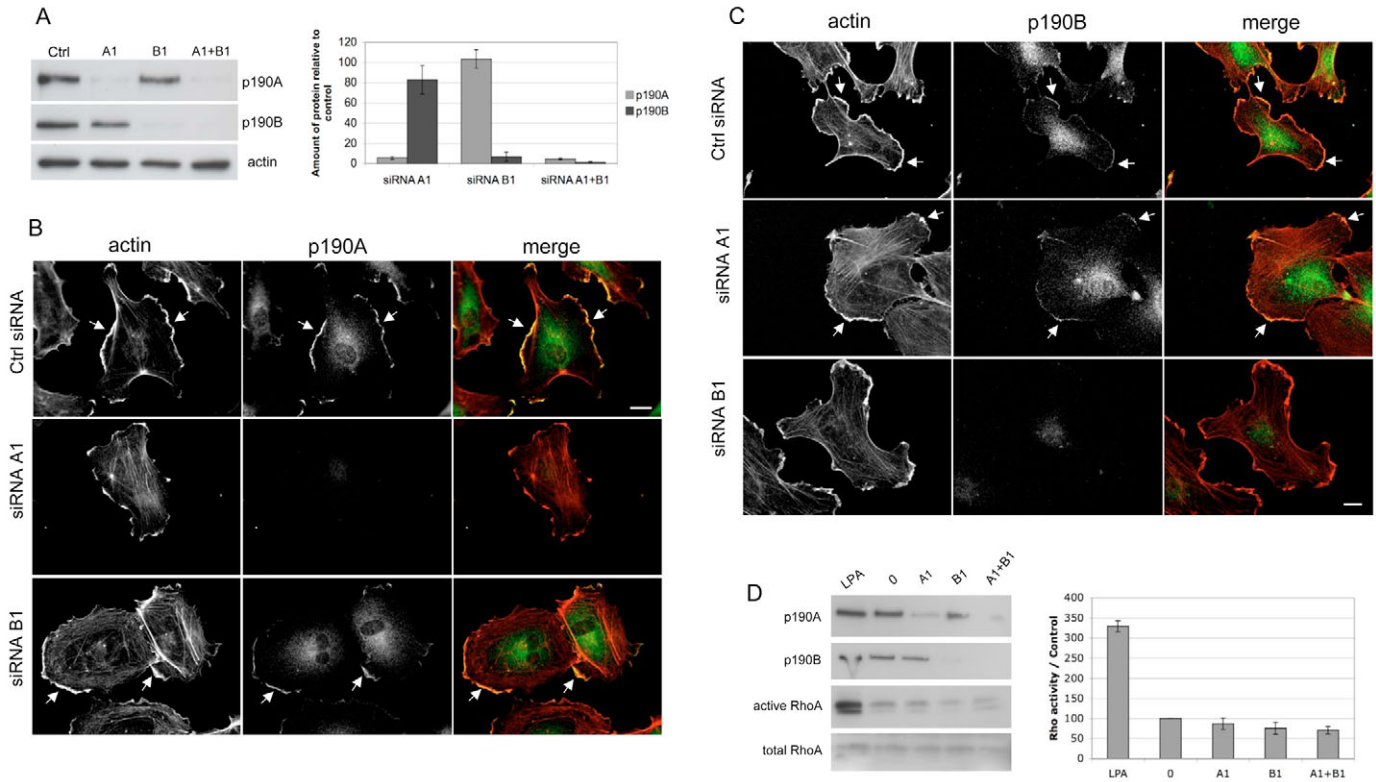


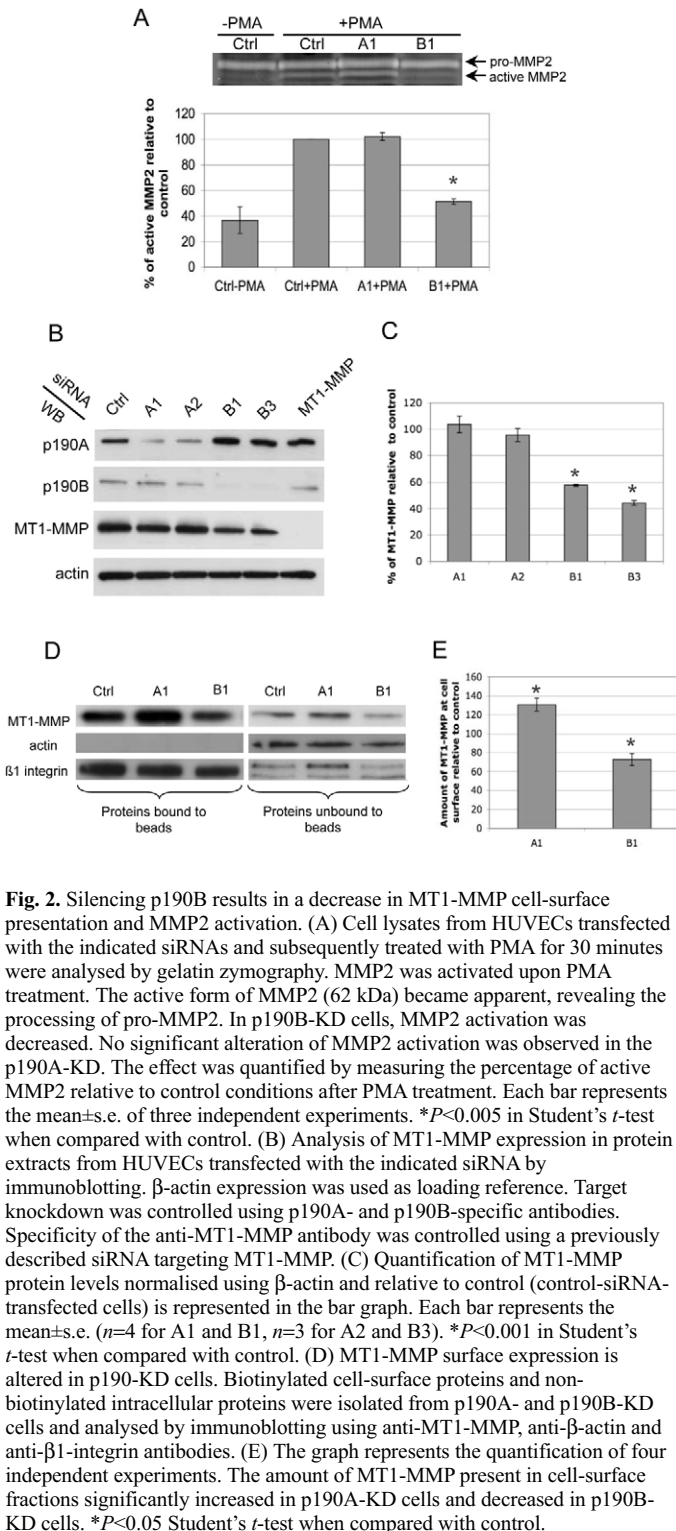
Fig. 1. (A) Specific siRNAs inhibit the expression of p190 isoforms in HUVECs. HUVECs were transfected with the indicated siRNAs targeting either p190A (A1), p190B (B1) or both (A1+B1). After 65 hours, protein extracts were analysed by immunoblotting using specific antibodies. The expression of endogenous β -actin remained unchanged. (Graph) The effect was quantified by measuring the relative amounts of p190 isoforms in each condition. Each bar represents the mean \pm s.e. of five independent experiments for A1 and B1, and of four independent experiments for A1+B1. (B,C) Specific siRNAs inhibit the expression of p190 isoforms in HUVECs and do not alter the actin cytoskeleton. HUVECs were transfected with the indicated siRNAs, fixed and stained for F-actin and p190A (B) or p190B (C). Scale bars: 10 μ m. (D) Silencing p190A and p190B does not modify RhoA activity in HUVECs. Cells treated with 2 μ g/ml of lysophosphatidic acid (LPA) for 15 minutes or transfected with the indicated siRNAs were lysed and active RhoA GTPase was affinity-precipitated with GST-RBD-rothekin, eluted from the beads and analysed by immunoblotting using anti-RhoA antibodies. For each condition, a fraction of the lysate was run to monitor the RhoGAP knockdowns and the amount of GTPase before precipitation. The graph shows the quantification of bands from two independent experiments; error bars represent the mean \pm s.e. RhoA activity was quantified in comparison to the negative control (control-siRNA-transfected cells). In p190-KD cells, basal levels of RhoA were not altered. LPA, which is a potent activator of RhoA, was used as a positive control in this experiment.

tumour metastasis or angiogenesis. Insoluble fibrin is one of the barriers that angiogenic endothelial cells must penetrate for neo-vascularisation. MT1-MMP (also known as MMP14) is a membrane-associated MMP that plays a crucial role in angiogenesis by virtue of its pericellular fibrinolytic activity. Besides its important contribution to ECM cleavage and broad-spectrum proteolytic capacities, MT1-MMP also acts as a membrane-anchored activator of the soluble MMP MMP2 (Itoh and Seiki, 2006). Both MT1-MMP and MMP2 regulate local ECM degradation at podosomes in endothelial cells (Tatin et al., 2006). Expression of MMP genes is transcriptionally regulated by a variety of extracellular factors, including cytokines and growth factors, as well as signals from the ECM.

Because RhoA regulates several steps of the angiogenic programme (Merajver and Usmani, 2005), we examined the role played by p190 isoforms in endothelial cells. Here, we provide data showing that the two p190 isoforms play distinct roles associated with endothelial cell invasiveness. We show that p190B knockdown selectively affects MT1-MMP and MMP2 expression and activity. We also demonstrated that p190B is involved in local ECM degradation at podosomes and capillary tube formation by endothelial cells.

Results

Mammalian p190 proteins are expressed as two highly related and ubiquitous isoforms. To knockdown each *p190* gene, we designed *p190A*- and *p190B*-specific small interfering RNAs (siRNA) outside the regions of identity. The possibility of having off-target effects was ruled out by using two siRNAs for each isoform (namely siRNA A1 and A2 for *p190A*, and siRNA B1 and B3 for *p190B*). These siRNAs inhibited the expression of their respective target with an inhibitory rate over 90% without affecting the expression of the other isoform, as assessed by western blot (Fig. 1A and data not shown). Specificity of the siRNAs was also confirmed by immunofluorescence (Fig. 1B,C). In control cells, p190A and p190B mainly localised to lamellipodia and ruffles (Fig. 1B,C). In cells treated with either A1 or B1 siRNA, the targeted isoform was no longer detectable, whereas the untargeted isoform remained localised at lamellipodia and ruffles. Thus, p190A knockdown did not alter p190B localisation and vice-versa. Phase-contrast observation and morphometric analysis of the siRNA-transfected cells did not reveal any detectable change when compared with controls (supplementary material Fig. S1A,B). Because p190 proteins are major RhoA regulators (Vincent and Settleman, 1999), we analysed the response of the actin cytoskeleton to p190 depletion.



Again, no major alteration in the organisation of the actin cytoskeleton was observed when comparing phalloidin staining of HUVECs transfected with either control or p190-targeted siRNAs (Fig. 1B,C, supplementary material Fig. S1C). Analysis of adhesive structures did not reveal any obvious alteration in vinculin distribution at focal adhesions in cells treated with p190 siRNAs (supplementary material Fig. S1D). However, further analysis is

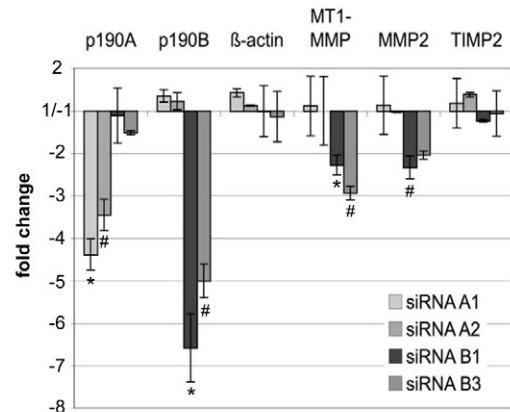


Fig. 3. Effect of p190 knockdown on MMP mRNA expression. Quantitative RT-PCR analysis of the effects of p190A and p190B knockdown on mRNA levels of p190A, p190B, MT1-MMP, MMP2, TIMP2 and β -actin in HUVECs. Each bar represents the mean±s.e. of four (A1, A2 and B1) or three (B3) independent experiments. * $P < 0.002$; # $P < 0.02$ in Student's *t*-test when compared with control.

required to fully exclude the absence of effect in F-actin and/or focal-adhesion turnover and stability in cells transfected with p190-targeted siRNAs. Moreover, as previously shown for p190^{-/-} mouse fibroblasts (Barberis et al., 2005; Brouns et al., 2000), siRNA-transfected HUVECs behaved similarly to their control counterparts in proliferation assays (data not shown). A redundant function for the closely related p190 isoforms could explain the absence of phenotype, but this hypothesis was ruled out when both isoforms were simultaneously knocked down (Fig. 1A). Indeed, cell morphology and behaviour remained unaltered under these conditions (supplementary material Fig. S1C and data not shown). In addition, RhoA-GTP basal levels measured with the Rhotekin Rho-binding domain (RBD) assay were not increased in these cells, as previously observed for p190A^{-/-} and p190B^{-/-} 3T3 fibroblasts (Wennerberg et al., 2003) (Fig. 1D). This latter result suggests that, in HUVECs, basal levels of RhoA activity are not regulated by p190 proteins or that other RhoGAPs compensate for the deficit in p190 proteins.

In search of a function for p190, we explored the effects of siRNA treatment specifically targeting each isoform on functions related to endothelial cell invasiveness. MMP2 activity plays a crucial role in angiogenesis and matrix remodelling. In vitro, MMP2 can be regulated by phorbol esters (Foda et al., 1996). Using gelatin zymography, HUVECs transfected with p190B-targeted siRNA (designated as p190B-KD cells) showed reduced levels of active MMP2 when compared with cells transfected with either control or p190A-targeted siRNA (designated as p190A-KD cells) in response to phorbol-12-myristate-13-acetate (PMA) treatment (Fig. 2A). Quantification of the results showed that p190B siRNA consistently inhibited MMP2 activation by 50% on average (Fig. 2A). Because processing of pro-MMP2 into active MMP2 is driven by the membrane-anchored MMP MT1-MMP, we also analysed the expression of MT1-MMP in HUVECs transfected with control, A1 or B1 siRNAs (Fig. 2B). Western blot experiments revealed that p190B-siRNA-mediated knockdown resulted in 40–55% reduction of MT1-MMP protein expression (Fig. 2C). By contrast, siRNA-mediated knockdown of p190A did not significantly alter MT1-MMP expression. Because the function of MT1-MMP requires its cell-surface

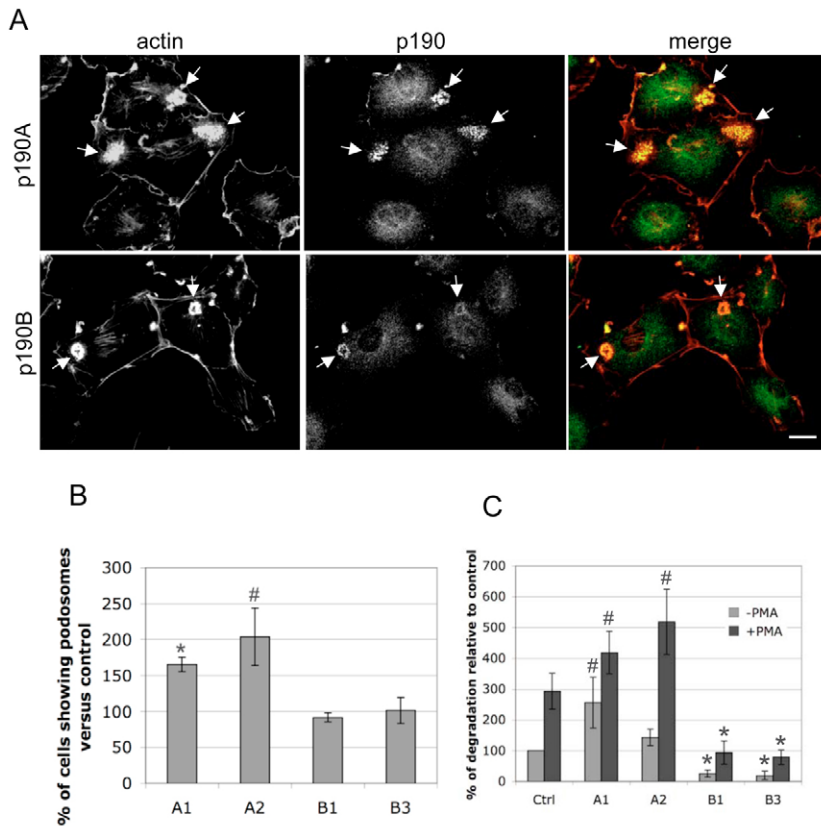


Fig. 4. Effect of p190 knockdown on podosome formation and function. (A) p190A and p190B localised to podosomes in HUVECs. F-actin was labelled using rhodamine-phalloidin and p190 isoforms were labelled using anti-isoform-specific antibodies and secondary Alexa-Fluor-488-conjugated antibodies in HUVECs treated with PMA. Arrows indicate podosome structures. Scale bar: 10 μ m. (B) Knockdown of p190B did not alter podosome formation, but knockdown of p190A increased the number of cells showing podosomes. HUVECs were transfected with siRNA designed against p190A or p190B for 65 hours, treated for 1 hour with PMA and stained for F-actin. Cells showing podosomes were counted and data are presented as a percentage compared with the control (control-siRNA-transfected cells). Error bars represent the mean \pm s.e. of ten (A1 and B1) or three (A2 and B3) independent experiments. * $P < 0.0005$; # $P < 0.05$ in Student's *t*-test when compared with control. (C) Alteration of in situ proteolytic activity in p190-KD cells. HUVECs transfected with the indicated siRNA were seeded on FITC-gelatin-coated coverslips. Half of the coverslips were treated with PMA for 1 hour to induce podosome formation. Cells were fixed and stained for F-actin. 'Black holes' representative of gelatin-degradation zones were recorded as described in the Materials and Methods section and are presented as a percentage of the control response obtained with untreated cells. Each bar represents the mean \pm s.e. of four independent experiments. The effects obtained were statistically significant (# $P < 0.05$; * $P < 0.01$ in Student's *t*-test) for p190A- and p190B-KD cells.

presentation, we then examined surface expression of MT1-MMP in these cells. Biotinylated cell-surface proteins were affinity-purified on streptavidin beads from a total-cell extract; unbound proteins, such as β -actin, are defined as the intracellular fraction (Fig. 2D). Fig. 2D,E show a decreased level of MT1-MMP at the surface of p190B-KD cells compared with control cells, consistent with the reduced activation of MMP2 in those cells. By contrast, p190A-KD cells displayed an increased amount of MT1-MMP on the cell surface. These results, confirmed by FACS analysis (supplementary material Table S1), suggest that p190 proteins antagonistically influence MT1-MMP cell-surface expression. This variation of MT1-MMP protein level at the cell surface might be explained either at the transcriptional or post-transcriptional level, or by alteration of MT1-MMP trafficking.

We examined the effect of p190 knockdown on MMP expression at the transcriptional level. MT1-MMP, MMP2, p190A, p190B, β -actin and the tissue-inhibitor of MMP2 (TIMP2) expression levels were measured by quantitative real-time PCR (qRT-PCR). Fig. 3 shows qRT-PCR results obtained from p190-siRNA-transfected HUVECs. We confirmed that, for each knockdown, paired siRNAs gave similar results and were isoform specific. We found that p190B-siRNA-based knockdown resulted in a significant reduction in the levels of MT1-MMP and MMP2 mRNA, with no alteration in TIMP2 mRNA expression. Thus, the decrease in MT1-MMP mRNA expression level correlated with the decrease of protein MT1-MMP present at the cell surface in p190B-KD cells. Interestingly, no such effects were observed at the mRNA level after p190A knockdown. These results indicate that p190A and p190B isoforms play distinct roles in endothelial cells. Our data revealed that p190B regulates MT1-MMP and MMP2 mRNA levels in the cell.

MT1-MMP and MMP2 are associated with local matrix degradation mediated by podosomes, and MMPs have been shown to alter podosome turnover (Goto et al., 2002). We therefore addressed the role of p190 in podosome formation and function. p190A has been shown to localise to podosomes in smooth muscle and porcine aortic endothelial cells (Burgstaller and Gimona, 2004; Moreau et al., 2006). We previously described that in HUVECs short-term exposure to PMA induced disruption of stress fibres followed by the appearance of podosomes and rosettes (Tatin et al., 2006); this defect was characterised by the presence of vinculin rings around actin dots (supplementary material Fig. S2). We first investigated p190A and p190B relocalisation in HUVECs. To visualise cytoskeletal structures, polymerised actin was stained with rhodamine-conjugated phalloidin. Co-staining of F-actin with p190 isoforms revealed that p190A and p190B, which both localised to lamellipodia in untreated HUVECs (Fig. 1B,C), relocalised to podosomes in response to PMA treatment (Fig. 4A). Moreover, similar results were observed in the two other podosome-forming cell lines, i.e. porcine aortic endothelial cells expressing constitutively active Cdc42 (Moreau et al., 2003) and A7r5 smooth muscle cells treated with phorbol esters (Hai et al., 2002) (supplementary material Fig. S3). We then analysed the impact of p190A or p190B knockdown on podosome formation in HUVECs. With this intention, p190A- or p190B-KD cells were treated for 1 hour with PMA to induce podosomes and were then stained for F-actin and the podosomal marker vinculin. The number of cells with podosomes was evaluated under the microscope. Fig. 4B shows the percentage of cells showing podosomes in p190-KD cells relative to control siRNA-transfected cells. Inhibition of p190A expression resulted in a significant increase in the number of cells with podosomes, whereas the absence of p190B had no effect on this

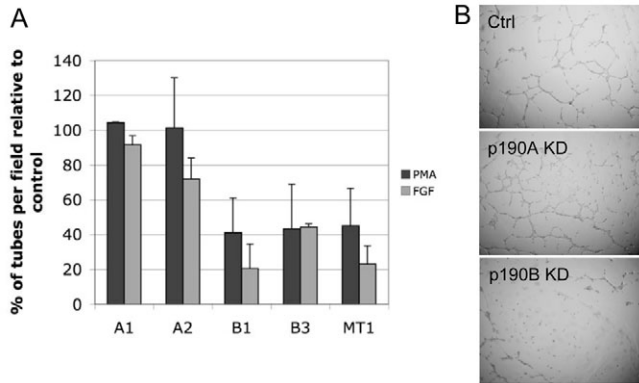


Fig. 5. Knockdown of p190B prevents PMA- and FGF-induced capillary tube formation in Matrigel. (A) HUVECs transfected with control, A1, A2, B1, B3 or *MT1-MMP* siRNA were seeded on Matrigel. The formation of PMA- and FGF-induced capillary tubes was quantified after 6 hours at 37°C by counting the number of tubes. The means and s.e. of the percentage of tubes per field relative to the control are shown ($n=3$). The formation of tubes induced by PMA or FGF was significantly inhibited ($P<0.05$ in Student's *t*-test) in p190B- and *MT1-MMP*-KD cells when compared with control (control-siRNA-transfected cells). (B) Representative photomicrographs of capillary-like structures from control-, A1- or B1-siRNA-transfected HUVECs cultured on Matrigel for 6 hours in the presence of PMA are shown.

parameter. To analyse the in situ proteolytic activity existing in these cells, fluorescent-matrix degradation assays were carried out. HUVECs were seeded onto glass coverslips pre-coated with FITC-conjugated crosslinked gelatin and treated or not for 1 hour with PMA. ECM degradation was quantified by counting the dark areas present in the fluorescent matrix (Fig. 4C). The absence of p190A significantly affected endogenous (no treatment) and podosome-induced (+PMA) degradation. The fact that p190A depletion modulates intrinsic matrix degradation suggests that the effect measured upon PMA addition reflects the effects of the knockdown both on podosome formation and function, i.e. matrix degradation. By contrast, the absence of p190B dramatically decreased matrix degradation in the absence (no treatment) or presence (+PMA) of podosomes. These experiments show that only p190A is involved in PMA-induced actin reorganisation and podosome formation, and that both p190 isoforms are required for matrix degradation in HUVECs, but in an antagonist manner. Thus, p190B might control matrix degradation, and it might do so via the regulation of MMP expression and activity.

The importance of the cooperation between MMP2 and *MT1-MMP* during angiogenesis has been well documented. Their role in the formation of new capillaries has been demonstrated in vitro and in vivo using numerous systems and various experimental procedures. Thus, anti-*MT1-MMP* antibodies have been shown to prevent the formation of capillary tubes by HUVECs on Matrigel

(Galvez et al., 2001; Genis et al., 2006). Because p190B plays a role in the regulation of *MT1-MMP* expression and matrix proteolysis, we set up in vitro assays using Matrigel to evaluate capillary tube formation by siRNA-treated HUVECs in response to fibroblast growth factor (FGF) or PMA (Fig. 5A,B). The stimulation of tube assembly by FGF and PMA in HUVECs was consistently inhibited by *p190B* siRNA, without interfering with cell adhesion to the matrix (Fig. 5 and data not shown). *p190A* siRNAs had no such effect, thus strengthening the functional differences between the two p190 isoforms in endothelial cells. *MT1-MMP* siRNAs also decreased tube formation in this assay, consistent with the inhibitory effect reported for anti-*MT1-MMP* antibodies (Genis et al., 2006). Our data demonstrate that the p190B isoform is selectively involved in matrix remodelling and in the resulting formation of capillary tubes by endothelial cells. We believe that this novel function of p190B arises from its involvement in the regulation of MMP expression.

Discussion

In this study, we investigated the roles of the p190 RhoGAPs in endothelial cells using siRNA-mediated gene-knockdown strategies. Both isoforms of p190 function as negative regulators of Rho activity. However, when p190A and/or p190B were knocked down in HUVECs, no increase in RhoA activity, nor any obvious alteration of the organisation of the actin cytoskeleton into stress fibres or of focal adhesions, were detected. These results suggest that, in HUVECs, other RhoGAPs might keep RhoA in its inactive form even in the absence of p190 proteins. Another explanation would be that p190 proteins control RhoA activity only locally and that alterations of this activity cannot be detected at the cell-population level. Even if the actin cytoskeleton of HUVECs remained unaltered under basal conditions, p190A, but not p190B, knockdown impacted the process of podosome assembly in response to PMA. This result is consistent with the fact that RhoA activity is required for podosome formation in HUVECs (Tatin et al., 2006). As previously reported by Burgstaller and Gimona (Burgstaller and Gimona, 2004), modifying local equilibrium between inactive and active RhoA might affect podosome formation. Indeed, p190A knockdown increased the number of cells showing podosomes and also the associated degradation activity. Because it has been suggested that the degradation activity is directly correlated to the half-life of the structures (Linder, 2007), the observed increase of these two parameters might in fact reflect a reduction in podosome turnover in p190A-KD cells. The microtubule cytoskeleton was shown to be involved in podosome dynamics in macrophages (Kopp et al., 2006); however, we were not able to detect any alteration in the pattern of α -tubulin in these modified cells. Thus, our data suggest that, even if p190A and p190B proteins share a high degree of homology and are involved in similar signalling cascades, they might control actin-cytoskeleton-based processes through distinct mechanisms.

Table 1. qRT-PCR primers used in this study

Gene	Accession number	Sense primer	Antisense primer
<i>p190A</i>	NM_004491	AGAAAGAGCCGGTTGGTTCAT	AACATAGCCAAAGAGGCCCTTACG
<i>p190B</i>	NM_001173	GCTGACTTGAGAATTGTCATGTGC	AAGGAAGGGTGAAAGAATAAGATCC
<i>MT1-MMP</i>	NM_004995	TGAGATCAAGGCCAATGTTTCG	AGTGATTTCAATTATGTTGCCATTGG
<i>MMP-2</i>	NM_004530	TTTGATGGCATCGCTCAGATC	GTCACAGTCCGCCAAATGAA
<i>TIMP2</i>	NM_003255	TTTGCAATGCAGATGTAGTGATCA	AAATGTCGTTTCCAGAGTCCACTT
β -actin	NM_001101	CGCGAGAAGATGACCCAGAT	ACAGCCTGGATAGCAACGTACAT
<i>GAPDH</i>	NM_002046	CACCCATGGCAAATCC	TGGGATTTCCATTGATGACAAG

The specific role of each p190 isoform was further demonstrated when the effects of siRNA on functions related to endothelial cell invasiveness were analysed. The activity of two matrix MMPs, MMP2 and MT1-MMP, which have both been implicated in endothelial cell function, was decreased only in the p190B-KD cells. Subsequently, *MMP2* and *MT1-MMP* mRNA expression levels were found decreased. The decrease in MMP2 protein levels (pro-MMP2 + active MMP2) was not as high as the decrease in *MMP2* mRNA levels, which might reflect a low turnover of the protein. Thus, during the time course of the experiment, mRNA levels decrease more rapidly than protein levels. In the case of MT1-MMP, the decrease in mRNA levels correlated with the decrease in protein levels. Thus, our results suggest that the two collaborative proteases MT1-MMP and MMP2 are regulated by the same signalling pathways and transcription factors, and that p190B is specifically involved. So far, it has only been demonstrated that two different transcription factors, GATA2 and EGR1 regulate the expression of MMP2 and MT1-MMP in response to a type I collagen matrix in endothelial cells (Haas, 2005), even if both can be activated by mitogen-activated protein kinase (MAPK). Our preliminary results suggest that Erk1/2 activity is not altered in p190-KD cells (V.M., unpublished data). It remains to be elucidated whether alterations in MMP2 and MT1-MMP expression can be explained by the regulation of the activity of a specific transcription factor by p190B. Recently, it was reported that p190 proteins are able to physically interact with the serum-responsive transcriptional regulator TFII-I (Jiang et al., 2005), but this interaction seems to occur with both isoforms and TFII-I has never been shown to regulate MMP transcription. Another possible link is the cAMP-responsive element-binding protein (CREB): CREB activity is reduced in p190B-deficient mice (Sordella et al., 2002) and its interaction with ATF1 plays a role in CRE-dependent expression of MMP2 in metastatic human melanoma cells (Xie et al., 1997).

The regulation of MMP activities by the p190B isoform in endothelial cells also reveals its crucial role in matrix degradation. This suggests that p190B might regulate cell invasion during angiogenesis *in vivo*. It is of interest to note that p190B has been shown to be required for cell invasion during virgin mammary gland development, another model of tissue remodelling and protease action. p190B might facilitate the invasion of the terminal end buds into the surrounding fat pad during ductal morphogenesis (Chakravarty et al., 2003), a process dependent on MMP2 activity (Wiseman et al., 2003). More recently, Vargo-Gogola et al. (Vargo-Gogola et al., 2006) demonstrated that overexpression of p190B in transgenic mice disrupts mammary gland architecture, showing a disorganised ductal tree with increased branching and altered stroma surrounding the mature ducts. Indeed, an increase in collagen is observed in the stroma surrounding the p190B-overexpressing terminal end buds, suggesting that p190B might also play a role in matrix deposition. Thus, this function of p190B in matrix remodelling does not seem to be restricted to the endothelium.

In this study, we showed that the knockdown of p190A did not produce the same effects as did the knockdown p190B on endothelial cells. Because the decrease in MT1-MMP at the cell surface in the intracellular fraction (quantification not shown) and in the total-cell extract were in the same range, our interpretation is that p190B knockdown alters the overall expression of MT1-MMP and not its trafficking and/or localisation at the plasma membrane. By contrast, on the basis of the result that p190A knockdown increased the amount of MT1-MMP at the cell surface and not in the total-cell extract, it is tempting to speculate that p190A might be involved in targeting

MT1-MMP to the plasma membrane. This hypothesis is further strengthened by the fact that p190A has been found to interact with Arf6 in a high-throughput yeast two-hybrid screen mapping protein interactions in *Drosophila melanogaster* (Formstecher et al., 2005). Thus, it is striking to notice that some of the obtained effects turned out to be antagonistic. Indeed, whereas p190B knockdown decreased MT1-MMP level at the cell surface of HUVECs, p190A knockdown increased it. We also observed this antagonism when the effects of the two knockdowns on podosome function were analysed. We believe that further experiments, which are required to strengthen this observation, will provide new insights into the possible crosstalk between both isoforms. To date, out of the few studies that report the role of p190 proteins in endothelial cells, all focused on p190A, which was found to be mainly involved in the regulation of endothelial permeability (Harrington et al., 2005; Holinstat et al., 2006; Mammoto et al., 2007). This process, which is based on the regulation of the endothelial cytoskeleton, required p190A GAP activity. p190A has been shown to act downstream of: (1) PKC δ to regulate basal endothelial barrier function (Harrington et al., 2005), (2) focal adhesion kinase (FAK) to restore endothelial barrier integrity after thrombin-mediated increase of endothelial permeability (Holinstat et al., 2006), and (3) angiotensin-II in order to protect against endotoxin-induced vascular leakage (Mammoto et al., 2007). In addition, it has also been demonstrated that p190A activity is necessary for directional retraction of endothelial cells in the presence of semaphorin (Barberis et al., 2005). In this latter situation, p190A might mediate local release of stable cell-substrate adhesions. In the present study, we demonstrate that p190B is involved in matrix remodelling, impacting matrix degradation at podosomes and endothelial cell assembly into tube-like structures in Matrigel. Therefore, it is now clear that both p190 isoforms play crucial but distinct roles in endothelial cell biology.

Interactions between cells and the ECM are mediated by: (1) integral membrane proteins – including integrins, which provide a link between the ECM and the actin cytoskeleton, and (2) extracellular proteinases and their inhibitors, which mediate local degradation of ECM components. Our work demonstrates that p190B represents a novel interface between actin-cytoskeleton and ECM remodelling. Because the acquisition of a motile and invasive phenotype is also an important step in the development of tumours and metastasis, it becomes worth considering p190B as a new player in cancer, involved not only in actin-based motility, but also in ECM remodelling in this context.

Materials and Methods

Materials

Monoclonal anti- β -actin antibodies, PMA, glutathione-Sepharose beads, DMSO, gelatin and various chemicals were from Sigma. Mowiol 4-88 was from Calbiochem. Rhodamine-phalloidin and Alexa-Fluor-488-labelled secondary antibodies were purchased from Molecular Probes. Antibodies against MT1-MMP were from Millipore (Lem-2/15.8), and those against p190A, p190B and β 1-integrin were from BD Biosciences. Antibodies against vinculin (hVIN-1) and α -tubulin were from Sigma.

Cell culture and cell stimulation

HUVECs were cultured in 100-mm dishes coated with 0.2% gelatin in endothelial cell basal medium supplemented with 'supplement pack' (Promocell). In all experiments, HUVECs were used between passages 2 and 5. Cell stimulation was achieved using PMA at 50 ng/ml. The porcine aortic endothelial (PAE) cell line expressing V12Cdc42 under the control of an IPTG-inducible promoter was established previously (David et al., 1999) and cultured in F12 medium (Ham F12; GIBCO BRL) supplemented with 10% heat-inactivated FCS, penicillin-streptomycin, 100 μ M hygromycin B and 500 nM puromycin. V12Cdc42 expression was achieved by adding 0.1 mM IPTG. A7r5 rat smooth muscle cells (ATCC) were grown in low-glucose (1 g/l) DMEM supplemented with 10% heat-inactivated FCS and penicillin-

streptomycin. Podosome induction was achieved by a 1-hour PMA (50 ng/ml) treatment. All cells were cultured at 37°C in a 5% CO₂ humidified atmosphere.

Measurement of RhoGTPase activity

pGEX-2T constructs containing rotekin-Rho-binding domain were kindly provided by Martin Schwartz (University of Virginia, Charlottesville, USA). HUVECs were grown in 140-mm dishes, treated with siRNA, lysed and protein extracts were used for pull-down assays as previously described (Ren et al., 1999; Sander et al., 1998). Antibodies against RhoA were from Santa Cruz.

siRNA transfection

siRNAs were chemically synthesised (Qiagen) and introduced into HUVECs (200 pmol) using a calcium-phosphate precipitation-transfection protocol. In order to reach maximum transfection efficiency in primary endothelial cells, two rounds of transfection were performed (24 hours apart). All experiments were then performed about 40 hours after the second transfection, i.e. 65 hours after the first one. The antisense-strand siRNA targeted against the human p190A isoform was based on published sequences (Barberis et al., 2005). For p190B, we designed and used 5'-AACGTGCAGCTGCATCTAAAT-3' and 5'-AATGAGAAGCATATCTGGTA-3', named B1 and B3, respectively. For MT1-MMP, the siRNA sequence has been published previously (Tatin et al., 2006). As control, we used the AllStars negative-control siRNA from Qiagen.

Immunofluorescence microscopy

Cells plated onto glass coverslips were prepared for immunofluorescence microscopy as previously described (Moreau et al., 2003). Fluorescent images were recorded on an Eclipse Nikon microscope using a 63× oil-immersion lens. Confocal images were captured on a Zeiss LSM510 confocal microscope. The images were processed using Adobe Photoshop 5.5 (Adobe Systems). Quantification of cells showing podosomes was assessed in three independent experiments in which at least 200 cells were counted.

ECM degradation assay

HUVECs were seeded on FITC-gelatin-coated coverslips prepared as described previously (Bowden et al., 2001). Briefly, a thin layer of fluorescein-conjugated gelatin was deposited on coverslips and cross-linked with 0.5% glutaraldehyde for 15 minutes at room temperature. After a wash in PBS and a 2-minute incubation in 70% ethanol, coverslips were kept in serum-free medium at 37°C until cells were seeded on the gelatin. For podosome-formation stimulation, 50 ng/ml PMA was added to cells in complete growth medium for 1 hour at 37°C. Cells were fixed in PFA and processed for immunofluorescence microscopy as described above. Colocalisation between dark areas and podosomes was visualized after merging FITC and rhodamine-phalloidin images. To quantify ECM degradation, we first determined the percentage of cells that presented at least one degradation area relative to the total number of cells analyzed in each condition. Values were then expressed as the percentage of degrading cells relative to control conditions (without PMA, transfected with control siRNA).

Analysis of matrix metalloproteinase activity by zymography

MMP activity was detected in cell supernatants and extracts as previously described (Tatin et al., 2006).

Biotinylation of cell-surface proteins

Cell-surface proteins were biotinylated as previously described (Remacle et al., 2003). Cells at 80% confluence in a 100-mm dish were incubated with 3.8 ml of a 1.5 mg/ml sulfo-NHS biotin (Pierce) in PBS solution for 30 minutes at 4°C with gentle shaking. Following quenching of biotinylation in PBS plus 100 mM glycine, and further incubation for 30 minutes at 4°C with gentle shaking, glycine solution was aspirated and rinsed twice with PBS. Cells were then lysed and biotinylated cell-surface fractions and intracellular fractions were separated and analysed by immunoblotting.

FACS analysis of cell-surface proteins

siRNA-treated HUVECs were detached with PBS containing 20 mM EDTA at 37°C, washed and resuspended in PBS containing 0.5% BSA. In total, 2.5×10⁵ cells were incubated with MT1-MMP (Lem-2/15) antibodies for 30 minutes at 4°C. Cells were washed with PBS and incubated with Alexa-Fluor-488-conjugated anti-mouse IgG. Finally, fluorescent samples were analysed in an EPICS-XL flow cytometer (Beckman COULTER).

Real-time PCR

RNA was collected from 6×10⁵ cells using the RNeasy mini kit (Qiagen). Integrity check and sample quantification were made on the 2100 bioanalyser (Agilent Technologies). cDNA was synthesised from 2 µg of total RNA with or without PowerScript reverse transcriptase (Clontech, Palo Alto, CA, USA) and with random primers (Invitrogen). Aliquots of cDNA were then subjected to PCR amplification on a DNA Engine Opticon2 fluorescence-detection system (MJ Research/Bio-Rad) with specific forward and reverse oligonucleotide primers (listed in Table 1). The DyNAmo SYBR Green qPCR kit (Finnzymes) was used with the following PCR amplification cycles: initial denaturation, 95°C for 15 minutes, followed by 40 cycles

with denaturation, 95°C for 20 seconds and annealing-extension, 61°C for 35 seconds. A dissociation curve was generated to verify that a single product was amplified. The cycle threshold (Ct) value of each gene was normalised against that of GAPDH and the relative level of expression calculated using the comparative (2^{-ΔΔCT}) method.

In vitro angiogenesis assay

Growth-factor-reduced Matrigel basement-membrane matrix (Becton Dickinson) was diluted 1:2 in endothelial growth medium. Diluted Matrigel (200 µl) was plated into flat-bottomed 48-well tissue-culture plates and allowed to gel for 45 minutes at 37°C before cell seeding. Then, 2.5×10⁴ cells were added atop the Matrigel in the presence of 20 ng/ml PMA or 10 ng/ml FGF. After 6 hours of incubation, images were taken with a phase-contrast microscope (Nikon TE 2000) using a 4× objective. Capillary tubes were defined as cellular extensions linking cell masses or branch points. The formation of capillary tubes was quantified by counting the numbers of tubes per field.

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