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Research Article

Tks5 recruits AFAP-110, p190RhoGAP, and cortactin for podosome formation

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ABSTRACT

Podosome formation in vascular smooth muscle cells is characterized by the recruitment of AFAP-110, p190RhoGAP, and cortactin, which have specific roles in Src activation, local down-regulation of RhoA activity, and actin polymerization, respectively. However, the molecular mechanism that underlies their specific recruitment to podosomes remains unknown. The scaffold protein Tks5 is localized to podosomes in Src-transformed fibroblasts and in smooth muscle cells, and may serve as a specific recruiting adapter for various components during podosome formation. We show here that induced mislocalization of Tks5 to the surface of mitochondria leads to a major subcellular redistribution of AFAP-110, p190RhoGAP, and cortactin, and to inhibition of podosome formation. Analysis of a series of similarly mistargeted deletion mutants of Tks5 indicates that the fifth SH3 domain is essential for this recruitment. A Tks5 mutant lacking the PX domain also inhibits podosome formation and induces the redistribution of AFAP-110, p190RhoGAP, and cortactin to the perinuclear area. By expressing a catalytically inactive point mutant and by siRNA-mediated expression knock-down we also provide evidence that p190RhoGAP is required for podosome formation. Together our findings demonstrate that Tks5 plays a central role in the recruitment of AFAP-110, p190RhoGAP, and cortactin to drive podosome formation.

Introduction

Podosomes are transient adhesion structures, which are composed of an F-actin-rich core that is surrounded by a ring structure containing various adhesion, scaffolding, and signalling components [1–3]. Podosomes have been identified in Src-transformed fibroblasts [4,5], osteoclasts [6,7], macrophages [8], dendritic cells [9], epithelial cells [10,11], endothelial cells [12,13], and vascular smooth muscle cells (vSMCs) [14–16]. Similar to the related invadopodia, podosomes are actively involved in focal degradation of extracellular matrix (ECM) components [13,17–21]. There is strong evidence that PKCs and the non-receptor tyrosine kinase Src are the major upstream activators of the signalling cascades leading to the formation of podosomes [19,22–28]. In cultured A7r5 vSMCs activation of conventional PKCs by contraction-inducing phorbol esters TPA and PDBu triggers the Src-dependent remodelling of the actin cytoskeleton and the formation of podosomes [15,29]. PKC-induced activation

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Abbreviations: vSMCs, vascular smooth muscle cells; ECM, extracellular matrix; PKCs, protein kinases C; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBu, phorbol-12,13-dibutyrate; AFAP-110, actin-filament-associated protein of 110 kDa; PH, pleckstrin homology domain; p190RhoGAP, Rho-GTPase-activating protein of 190 kDa; N-WASp, neural Wiskott-Aldrich Syndrome protein; PX, Phox homology domain; SH3, Src homology 3 domain

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of Src has been proposed to trigger a spatio-temporally restricted modulation of actin cytoskeleton stability and dynamics, including the local down-regulation of RhoA activity, which results in disassembly of stress fibres and focal adhesions, and in the induction of Arp2/3-dependent de novo actin polymerization at the sites of podosome formation. Key molecules in this double-faceted process are the podosome components AFAP-110, p190RhoGAP, and cortactin.

AFAP-110 is an adaptor protein that contains two PH domains and a binding site for Src [30,31]. The molecule translocates from stress fibres to sites of podosome formation in stimulated A7r5 cells [32], and is essential for mediating PKC-dependent activation of Src and the subsequent formation of podosomes [28]. Upon phosphorylation by Src, p190RhoGAP (the p190A isoform) down-regulates RhoA activity, resulting in the inhibition of actin stress fibre and focal adhesion formation [33–35]. F-actin and p190RhoGAP co-distribute also in invadopodia, and microinjection of antibodies directed against p190RhoGAP inhibits localized ECM degradation [36]. Moreover, in response to phorbol esters p190RhoGAP is tyrosine-phosphorylated and recruited to the sites of podosome formation, where it may down-regulate RhoA activity [29,32]. The actin-binding protein cortactin is phosphorylated by Src [37,38] and interaction with N-WASp and the Arp2/3 complex induces branched actin polymerization, which is essential for both invadopodia and podosome formation, and for their ability to degrade the ECM [39]. Cortactin clusters at the sites of podosome formation in PDBu-stimulated A7r5 cells, and we have demonstrated earlier that small cortactin clusters are present at the actin stress fibre/focal adhesion interface also in unstimulated A7r5 cells [32]. Cortactin is necessary to promote podosome formation, and siRNA-mediated suppression of cortactin expression inhibits podosome formation in PDBu-stimulated A7r5 cells [40].

While AFAP-110, p190RhoGAP, and cortactin are critically involved in the regulation of podosome formation, the molecular mechanism that underlies their specific recruitment to sites of podosome formation remains largely unknown. Spatio-temporally regulated recruitment and regulation of signalling components is largely driven by a variety of modular scaffolding proteins. The adaptor protein Tks5, which contains one PX domain, five SH3 domains, and three canonical poly-proline motifs, is tyrosine-phosphorylated by Src [41], and localizes to podosomes in Src-transformed fibroblasts [42]. Importantly, Tks5 is required for podosome formation, ECM degradation, and tissue invasion [43,44], and for invadopodium formation in human cancer cells [43]. Tks5 is thus a likely candidate for acting as a key scaffolding molecule in podosome forming cells. In this study, we addressed the hypothesis that Tks5 acts to specifically recruit AFAP-110, isoform A of p190RhoGAP, and cortactin to sites of podosome formation. We induced subcellular mistargeting of Tks5 to the surface of mitochondria by fusing the membrane anchor of the *Listeria monocytogenes* surface protein ActA to the C-terminus of a GFP-tagged Tks5 construct (GFP-Tks5-mito). When expressed in eukaryotic cells, the membrane anchor of ActA displays a selective affinity for the mitochondrial membrane [45]. This approach has already been used successfully to mistarget vinculin [46], zyxin [47,48], and the actin-binding protein CRP-2 [49] to the surface of mitochondria. Since AFAP-110, p190RhoGAP, and cortactin do not show mitochondrial localization under normal conditions, this approach is suitable to determine the recruitment potential of Tks5. Here we show that Tks5-dependent recruitment and clustering of podosome components is critically involved in integrating and coordinating the restricted down-regulation of RhoA activity with Arp2/3-dependent polymerization of actin filaments in the podosome core.

Materials and methods

Antibodies and reagents

Monoclonal antibodies to AFAP-110 and p190RhoGAP were from BD Biosciences, monoclonal anti-cortactin was from Upstate

Table 1 – List of GFP constructs.

GFP-Tks5-mito mutant	Residues deleted	5′–3′ sequence of the primer pairs[a]
GFP-Tks5(ΔPX)-mito	6–121	ATGCTCGCCTACTGCGAGGCTCGACCCGAG
		CTCGGGTCGAGCCTCGCAGTAGGCGAGCAT
GFP-Tks5(ΔSH3#1)-mito	154–209	GAGCCCATGATCCTGGGTACTCGGGATGAC
		GTCATCCCGAGTACCCAGGATCATGGGCTC
GFP-Tks5(ΔSH3#2)-mito	254–309	CAGCACAGCCGAGAGGATGACCTGCCAACC
		GGTTGGCAGGTCATCCTCTCGGCTGTGCTG
GFP-Tks5(ΔSH3#3)-mito	436–491	CCCCCTTCTGTTGAGAAGCCCAACCTGAGC
		GCTCAGGTTGGGCTTCTCAACAGAAGGGGG
GFP-Tks5(ΔSH3#4)-mito	828–883	TGGGAAGGGCCAGCCAACGAGCAACCTGAC
		GTCAGGTTGCTCGTTGGCTGGCCCTTCCCA
GFP-Tks5(ΔSH3#5)-mito	1060–1118	CACAATAACCTCAAAGGAAGCTTCGAATTC
		GAATTCGAAGCTTCCTTTGAGGTTATTGTG
GFP-Tks5(ΔPP1)-mito	426–433	CTGGGGTTCCAACTGGTTGAGGTGGAGTAC
		GTACTCCACCTCAACCAGTTGGAACCCCAG
GFP-Tks5(ΔPP2)-mito	505–516	AGCACGCTGACCCGGAAGGAGGCCGAGGAG
		CTCCTCGGCCTCCTTCCGGGTCAGCGTGCT
GFP-Tks5(ΔPP3)-mito	932–939	AGCAAGAAGGCCACGGGGGGCTTCGGCAAG
		CTTGCCGAAGCCCCCCGTGGCCTTCTTGCT

[a] Top row: forward primer; Bottom row: reverse primer.