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Cdc42-driven podosome formation in endothelial cells

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Abstract

Ectopic expression of a constitutive active mutant of the GTPase Cdc42 (V12Cdc42) in vascular endothelial cells triggers the dissolution of stress fibres and focal adhesion contacts and causes the repolymerisation of actin into dots. Each punctate structure consists of an F-actin core surrounded by a vinculin ring, consistent with the definition of podosomes. We now report further analysis of these complexes and show the presence of established podosomal markers such as cortactin, gelsolin, dynamin, N-WASP, and Arp2/3 which are absent in focal adhesions. Endothelial podosomes appear as randomly distributed conical structures, distributed on, but restricted to, the ventral membrane and confined to contact sites between cells and their substratum. The nature of the extracellular matrix does not influence podosome formation nor their spatial organisation. Induction of podosomes in response to V12Cdc42 is not associated with a migratory nor with a proliferative phenotype. These results add endothelial cells to the list of cell types endowed with the ability to form podosomes in vitro and raise the possibility that endothelial cells could form such structures under certain physiological or pathological conditions.
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Keywords: Podosomes; Endothelial cells; Cdc42; GTPases; F-actin

Introduction

Cytoskeletal rearrangements are central to endothelial cell physiology and are controlled by soluble factors, matrix proteins, cell–cell interactions and mechanical forces. Within the endothelium lining the inner surface of blood vessels, endothelial cells are subjected to haemodynamic forces caused by blood flow and dilate or contract in response to vasoactive substances. Cytoskeletal plasticity is also required for correct extravasation of blood-borne leucocytes at sites of inflammation. Cytoskeleton organisation is affected when cell–cell interactions are disrupted at the initiation of the angiogenic program, when cells detach from the endothelium and then proliferate and migrate to extend the preexisting vascular network. Such abundance and diversity of signals impact on cytoskeletal organisation and produce a wide range of actin configurations, which therefore represent a critical aspect of endothelial cell physiology. Podosomes form spontaneously in certain cell types of haematopoietic origin, such as monocytes/macrophages and osteoclasts (Linder and Aepfelbacher, 2003). The hemangioblast being a common progenitor to blood and endothelial cells (Choi et al., 1998), we investigated whether endothelial cells share the ability to form podosomes with these cells of haematopoietic origin. We report that a constitutively active mutant of Cdc42 but not its wild-type or dominant negative forms

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induces the formation of podosomes. We describe V12Cdc42-induced podosomes and certain aspects of cell behaviour observed in these podosome-bearing cells.

Materials and methods

Reagents and antibodies

Puromycin, hygromycin B, isopropyl-beta-D-thiogalactopyranoside (IPTG) and Mowiol 4–88 were from Calbiochem. Collagen type I and fibronectin were from BD Biosciences. Antibodies against vinculin (hVIN-1) were from Sigma. Anti-myc (9E10), anti-phosphotyrosine (4G10) and anti-p85α (U5) antibodies were kind gifts from Dr. D. Cantrell (University of Dundee, Dundee, UK). Antibodies against gelsolin were kindly supplied by Dr. C. Chaponnier (University of Geneva, Geneva, Switzerland). Polyclonal anti-Arp3 antibodies were from Dr. M. Welch (University of California, Berkeley, USA), anti-dynamin 2 antibodies from Dr. M. McNiven (Rochester, USA), and anti-N-WASP antibodies from Dr. M. Way (London Research Institute, London, UK). Further antibodies were against cortactin (clone 4F11) (Upstate Biotechnology International), Cdc42, beta3 integrin, p190RhoGAP, VASP (Cell Signalling Technologies). WIP-myc tag construct was from D. Stewart (Bethesda, USA). Rhodamine-phalloidin and FITC-labeled secondary antibodies were purchased from Molecular Probes. Propidium iodide was from Sigma.

Cell culture

Porcine aortic endothelial (PAE) cells obtained from Dr. Saklatvala (Kennedy Institute of Rheumatology, London, UK) were maintained in F12 medium (Ham F12; GIBCO BRL) supplemented with 10% heat-inactivated FCS (Globepharm) and antibiotics. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. PAE cell lines expressing either V12, N17 or WTCdc42 under the control of an IPTG-inducible promoter were established previously (Davis et al., 1999) and cultured in the same medium supplemented with 100 μM hygromycin B and 500 nM puromycin. Expression was achieved by adding 0.1 mM IPTG to the cultures.

Immunofluorescence microscopy

Cells plated onto glass coverslips were prepared for immunofluorescence microscopy as previously described (Moreau et al., 2003). The coverslips were washed in water and mounted on microscope slides using Mowiol 4–88 mounting medium. Images were acquired using a TE2000 Nikon inverted fluorescence microscope and a Nikon DXM 1200 camera (LUCIA 5.0 acquisition software, Nikon). Confocal images were captured on an Eclipse E800 Nikon microscope. The images were processed using Adobe Photoshop 5.5 (Adobe Systems). The number of cells showing podosomes was assessed in three independent experiments in which at least 200 cells were counted.

Wound healing assay

Endothelial cells were seeded in six-well tissue culture plates and grown to confluence. The cell monolayer was wounded with a sterile plastic pipette tip to generate a wound with a width of approximately 1 mm. The cell monolayer was washed with PBS to remove cell debris and incubated in complete medium at 37 °C. Images were taken using a phase-contrast microscope with a 5× lens, directly after wounding and after a 10-h incubation period. Cell migration was determined by counting the number of cells within the wound area. Experiments were done in triplicate and four fields from each well were randomly selected for cell counting. The percentage of migrating cells was determined and is presented as percentage of the control response.

Results and discussion

Cytoskeletal arrangements, including podosomes, are controlled by members of the Rho family of GTPases. By analysing the effects of ectopic expression of constitutively active mutants of this class of proteins on F-actin organisation, we discovered that endothelial cells can form podosomes. Using inducible PAE cell lines (Davis et al., 1999) we show that only overexpression of active (V12Cdc42), but not wild-type (WTCdc42) nor dominant negative (N17Cdc42) Cdc42, triggered podosome formation (Fig. 1A). In this model, podosomes appeared scattered all over the ventral cell surface (Fig. 1A). At higher magnification the characteristic podosomal protein organisation consisting in a core of F-actin surrounded by a ring of vinculin could be seen (Fig. 1B). Interestingly, filopodia-like protrusions could also be observed at the periphery of V12Cdc42-expressing PAE cells and elongated focal complexes had replaced the classical focal adhesions (Fig. 1B).

In a time-course experiment analysing actin polymerisation upon V12Cdc42 transgene expression, we observed the first podosomes as early as 4 h after induction, when V12Cdc42 protein became detectable by Western blot (data not shown). At 24 h, about 25% of the cells had flattened, lost their stress fibres and presented isolated or scattered podosomes. By 48 h, about half of the cells had increased in volume and

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