Caldesmon is an integral component of podosomes in smooth muscle cells

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Summary

Podosomes are highly dynamic actin-based structures commonly found in motile and invasive cells such as macrophages, osteoclasts and vascular smooth muscle cells. Here, we have investigated the role of caldesmon, an actin-binding protein, in the formation of podosomes in aortic smooth muscle A7r5 cells induced by the phorbol ester PDBu. We found that endogenous low molecular weight caldesmon (l-caldesmon), which was normally localised to actin-stress fibres and membrane ruffles, was recruited to the actin cores of PDBu-induced podosomes. Overexpression of l-caldesmon in A7r5 cells caused dissociation of actin-stress fibres and disruption of focal adhesion complexes, and significantly reduced the ability of PDBu to induce podosome formation. By contrast, siRNA interference of caldesmon expression enhanced PDBu-induced formation of podosomes. The N-terminal fragment of l-caldesmon, CaD40, which contains the myosin-binding site, did not label stress fibres and was not translocated to PDBu-induced podosomes. Cad39, the C-terminal fragment housing the binding sites for actin, tropomyosin and calmodulin, was localised to stress fibres and was translocated to podosomes induced by PDBu. The caldesmon mutant, CadCamAB, which does not interact with Ca^{2+}/calmodulin, was not recruited to PDBu-induced podosomes. These results show that (1) l-caldesmon is an integral part of the actin-rich core of the podosome; (2) overexpression of l-caldesmon suppresses podosome formation, whereas siRNA knock-down of l-caldesmon facilitates its formation; and (3) the actin-binding and calmodulin-binding sites on l-caldesmon are essential for the translocation of l-caldesmon to the podosomes. In summary, this data suggests that caldesmon may play a role in the regulation of the dynamics of podosome assembly and that Ca^{2+}/calmodulin may be part of a regulatory mechanism in podosome formation.

Key words: Actin cytoskeleton, Caldesmon, Podosome, Vascular smooth muscle

Introduction

Podosomes are highly dynamic actin-based structures that function as sites of cell adhesion and active extracellular matrix remodelling, which were originally described in highly motile, invasive cells such as macrophages, osteoclasts and transformed fibroblasts (Linder and Aepfelbacher, 2003). Recently, Src and phorbol ester have been shown to induce the formation of podosomes in endothelial cells (Moreau et al., 2003; Osiak et al., 2005) and vascular smooth muscle cells (Fultz et al., 2000; Hai et al., 2002; Gimona et al., 2003). Discovery of podosomes in an increasingly wider spectrum of invasive cell types underscores their importance and suggests a general requirement of these less well-characterised cytoskeletal organelles in cell invasion and migration. Podosomes share common architectural features but, depending on the cell types, vary in their molecular components and regulation. A typical podosome is defined by a finger-like vertical column arising from the ventral membrane that is in contact with the substrate and extending towards the dorsal cell surface (Linder and Aepfelbacher, 2003). The core of the podosome column (0.5-2 μm in diameter and 2-4 μm in height) comprises actin filaments and harbours a battery of actin polymerising proteins such as N-WASp (Mizutani et al., 2002), Arp2/3 (Linder et al., 2000; Burns et al., 2001; Kaverina et al., 2003) and cortactin (Schuuring et al., 1993; Ochoa et al., 2000; Pfaff and Jurdic, 2001; Mizutani et al., 2002; Destaing et al., 2003). The actin core is often surrounded by an outer sheath containing focal adhesion proteins such as α-actinin and vinculin (Marchisio et al., 1984; Tarone et al., 1985; Marchisio et al., 1988; Sobue et al., 1989; Babb et al., 1997; Fultz et al., 2000; Hai et al., 2002; Destaing et al., 2003). Podosomes are highly dynamic structures with a life time of 2-10 minutes, continuously assembled and disassembled by unknown mechanisms at sites near or at the end of stress fibres abutting focal adhesion complexes (Kaverina et al., 2003; Burgstaller and Gimona, 2004). Assembly of podosomes appears to involve: (1) recruitment of the actin polymerisation machinery, including monomeric G-actin, N-WASp, Arp2/3 complex and cortactin to the sites of podosome-formation; (2) polymerisation of actin to form actin bundles at the core; (3) recruitment of focal adhesion proteins, such as vinculin, to a ring surrounding the actin core; and (4) assembly of proteins required for functions specific for the cell type, e.g. MMPs for digestion of extracellular matrix and cell invasion (Linder and Aepfelbacher, 2003; Buccione et al., 2004; Linder and Kopp, 2005). At late stages of development, podosomes mature to higher order structures, and depending on the cell type, forming clusters, wide bands or rosettes of large numbers of individual podosomes.

The physiological function of podosomes remains unclear

and can only be inferred from circumstantial evidence. The general belief that podosomes play a major role in cell invasion stems from several lines of evidence: (1) podosomes in cultured cells localise to sites of active digestion of substrates (Chen and Wang, 1999; Mizutani et al., 2002); (2) localisation of MT1-MMP and MMP-9 to podosomes in osteoclasts (Sato et al., 1997; Goto et al., 2002; Anderson et al., 2004); (3) tubular membrane invagination has been described at the centre of some podosomes and is thought to be sites of MMP secretion (Ochoa et al., 2000); and (4) most recently, PDBu-induced podosomes in smooth muscle A7r5 cells and primary VSMC are sites of substrate degradations (A.S.M. et al., unpublished) (Burgstaller and Gimona, 2005). Other studies suggest that podosomes may also be involved in active cell migration by providing strong attachment sites to the ECM. For example, osteoclasts and macrophages that are defective in podosome formation are also found to be defective in motility (Linder et al., 1999).

Caldesmon is an actin-binding protein present in both smooth muscle and non-muscle cells (for reviews, see Matsumura and Yamashiro, 1993; Wang, 2001; Dabrowska et al., 2004). The heavier smooth muscle caldesmon (h-caldesmon, 87 kDa) is found in differentiated 'contractile' VSMC, whereas the lighter isoform (l-caldesmon, 57 kDa) is expressed in both non-muscle cells and 'synthetic' smooth muscle cells either in primary cell culture (Ueki et al., 1987; Birukov et al., 1993) or in the intimal thickenings (Reckless et al., 1994; Sobue et al., 1999). L-caldesmon consists of two major functional domains: an N-terminal domain containing binding sites for myosin (Velaz et al., 1990; Bogatcheva et al., 1993; Redwood and Marston, 1993; Huber et al., 1995) and tropomyosin (Smith et al., 1987; Redwood and Marston, 1993; Redwood et al., 1993); and a C-terminal domain housing the binding sites for actin, tropomyosin and calmodulin (Wang et al., 1991; Zhan et al., 1991; Marston et al., 1994; Wang et al., 1996; Wang et al., 1997a; Marston et al., 1998; Huang et al., 2003). There is strong in vitro evidence implicating caldesmon in the regulation of smooth muscle contraction and cell motility. Caldesmon inhibits the actin-activated myosin ATPase (Dabrowska et al., 1985; Smith et al., 1987; Horiuchi and Chacko, 1989; Chalovich et al., 1998) by blocking the interaction of actin and myosin (Chalovich et al., 1998; Sen et al., 2001) and/or inhibiting a kinetic step of the actomyosin ATPase cycle (Marston and Redwood, 1993; Marston et al., 1998). Inhibition of contraction by caldesmon can be released by binding of caldesmon to Ca^{2+}/calmodulin and/or post-translational phosphorylation. In non-muscle cells and smooth muscle cells in culture, l-caldesmon plays a dual role in the regulation of cytoskeleton organisation by (1) modulating contractility (Helfman et al., 1999), and (2) stabilisation of the stress fibres and focal adhesions (Li et al., 2004; Ishikawa et al., 1998). By virtue of its involvement in cytoskeletal organisation, l-caldesmon plays a key role in cell movement (Helfman et al., 1999; Li et al., 2004), intracellular granule movement (Hegmann et al., 1991) and cytokinesis (Warren et al., 1996; Yamashiro et al., 2001).

We have recently shown that overexpression of p21-activated protein kinase (PAK) induced the formation of podosomes that shared common molecular and morphological features with PDBu-induced podosomes in A7r5 cells and that caldesmon was localised at the actin core of podosomes (Webb et al., 2005). Our data corroborates an early report (Tanaka et al., 1993) that showed localisation of caldesmon in podosomes in Src-transformed non-muscle cells. These results strongly implicated a crucial involvement of caldesmon in podosome formation and cell invasion, and prompted us to investigate further this unexplored function of caldesmon by determining the role of different functional domains of caldesmon in the assembly of podosomes in the vascular smooth muscle A7r5 cells. In this study, we show that: (1) l-caldesmon is translocated to the actin core of PDBu-induced podosomes in A7r5 cells as a result of its interaction with actin and calmodulin, but not with tropomyosin or myosin; (2) over-expression of l-caldesmon dissociates actin stress fibres and focal adhesion complexes, and reduces the ability of PDBu to induce podosome formation; and (3) by contrast, siRNA interference of l-caldesmon expression augments podosome formation. These results provide new evidence that caldesmon, although an integral component of the actin core of podosomes, plays a crucial role in the regulation of podosome dynamics by either inhibiting the assembly and/or enhancing the turn-over of podosomes.

Results

Endogenous l-caldesmon is recruited to phorbol ester-induced podosomes in smooth muscle cells

The A7r5 smooth muscle cells contain a prominent array of straight and parallel actin stress fibres in the bulk of the cell body and relatively few actin-containing lamellipodia at distal ends of the cell, as shown by immunofluorescence staining with TRITC phalloidin (Fig. 1A). Western blot analyses using a monoclonal antibody that recognises both h- and l-caldesmon detected the presence of l-caldesmon in A7r5 cell lysates, but not h-caldesmon, which is expressed in differentiated rat aortic smooth muscle cells (Fig. 1D). Immunofluorescent staining of cells with the same caldesmon antibody showed that l-caldesmon co-localised with the actin stress fibres, as well as the actin meshwork in the lamellipodia.

After treatment with PDBu for 30 minutes, about 50% of the cells had disrupted stress fibres and many developed podosomes in areas where parallel arrays of stress fibres became disorganised (Fig. 1B). The PDBu-induced podosomes were characterised by a vertical column of phalloidin-stained actin bundles spanning from the ventral to the dorsal cell surfaces (Fig. 1C) and contained proteins involved in actin polymerisation such as Arp2/3, N-WASp and cortactin (data not shown) in agreement with previous reports (Hai et al., 2002; Kaverina et al., 2003; Gimona et al., 2003; Burgstaller and Gimona, 2004; Webb et al., 2005). Caldesmon was clearly enriched in the podosomes and co-localised with the actin columns as revealed by cross-sections of podosomes doubly stained with anti-caldesmon antibodies and phalloidin (Fig. 1B,C). In areas where stress fibres remained intact and lacked podosomes, caldesmon remained localised to the actin fibres, suggesting that during the process of podosome genesis, actin and caldesmon were dissociated from pre-existing stress fibres and re-organised into vertical caldesmon-actin columns. This is further illustrated by video images showing the dynamics of PDBu-induced formation of podosomes in A7r5 cells co-transfected with EGFP-l-caldesmon and DsRed-cortactin, which was used to mark podosomes (see below). The video images showed that l-caldesmon and cortactin were both

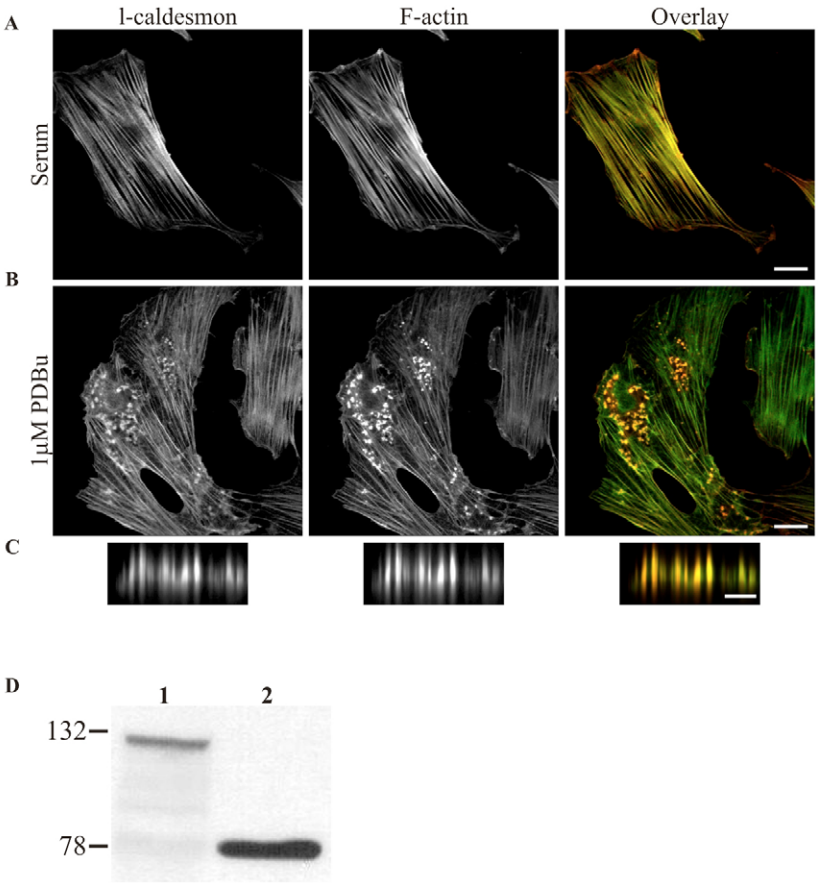


Fig. 1. Localisation of endogenous l-caldesmon in PDBu-induced podosomes in A7r5 vascular smooth muscle cells. A7r5 cells cultured on glass coverslips and were either unstimulated (A) or stimulated with 1 μM PDBu for 30 minutes (B). Cells were stained with antibodies raised against l-caldesmon and TRITC-phalloidin was used to label F-actin. The two images were merged (overlay) to show the relative localisation of l-cadesmon (green) and F-actin (red). (A) A7r5 cells grown in serum displayed a robust actin cytoskeleton with prominent stress fibres. (B) PDBu-stimulation induced the formation of podosomes containing punctate actin staining. (C) Reconstruction of the x-z profiles of typical podosomes in B; caldesmon co-localised with the actin-rich core of the podosome. Bars, 20 μm in A,B; 2 μm in C. (D) Western blot analysis of rat aortic smooth muscle cell lysate (lane 1) or A7r5 cell lysate (lane 2) probed with a general caldesmon antibody. Whereas aortic smooth muscle expresses the high molecular weight isoform of caldesmon (h-caldesmon), cultured A7r5 cells express the low molecular weight isoform (l-caldesmon). The molecular masses of protein standards (in kDa) are listed on the left.

recruited to and enriched in podosomes less than 5 minutes after PDBu treatment.

Effects of expression of EGFP-caldesmon on the cytoskeleton organisation and podosome formation in smooth muscle cells

To determine the role of caldesmon in podosome genesis, we studied the effects of overexpressing EGFP-l-caldesmon, the EGFP-tagged wild type l-caldesmon, in PDBu-induced formation of podosomes (Fig. 2). It has been previously reported that overexpression of caldesmon in non-muscle cells caused disorganisation of actin stress fibres and focal adhesion complexes as a result of inhibition of cell contraction by the exogenous caldesmon (Helfman et al., 1999). In view of these findings, it is necessary to first establish whether overexpression of caldesmon in smooth muscle cells also affects the actin cytoskeletal organisation, and may induce podosome formation even in the absence of phorbol ester stimulation. As shown in Fig. 3B,D, transient transfection of A7r5 cells with EGFP-l-caldesmon caused a disassembly of stress fibres and focal adhesions. Transfection for 48 hours induced disassembly of stress fibres in about 40% of the transfected cells, where distribution of actin and exogenous caldesmon became diffuse and often accompanied by development of extended processes (Fig. 3B,D). The remaining 60% of the cells, which expressed lower levels of the exogenous protein, appeared to be relatively unaffected showing caldesmon-associated actin stress fibres (Fig. 3C,E). Vinculin-staining showed that prominent and large 'fish-eye' type of focal adhesion complexes were distributed both in the interior and periphery of the non-transfected cells (Fig. 3D).

To determine if there is a correlation between the expression level of exogenous l-Cad and its effect on stress fibre disorganisation, we first compared the expression level of EGFP-l-caldesmon at 20 hours and 48 hours of transfection. As shown in Fig. 3A, western blot analyses showed that the expression level of exogenous EGFP-l-caldesmon increased by about fivefold between 20 hours and 48 hours of transfection (Fig. 3A) and correlated with the severity of stress fibre disruption (15% of transfected cells had disrupted stress fibres at 20 hours transfection versus 47% at 48 hours, data not shown). This is further illustrated by comparing the staining intensity of EGFP-l-caldesmon and stress fibre organisation in individual cells 48 hours after transfection. As shown in Fig. 3B-E, in cells expressing a high level of caldesmon (cell 1 in Fig. 3D), focal adhesions were few and small, accompanying a diffuse distribution of caldesmon. Cells that expressed medium (cell 2, Fig. 3E) and low levels (cell 3, Fig. 3E) of caldesmon retained some stress fibres and less disrupted focal adhesions. By comparison, overexpression of EGFP-l-caldesmon for 48 hours in 3T3 fibroblasts caused severe collapse of the actin cytoskeleton in over 90% of the transfected cells (data not show) in agreement with a previous report (Helfman et al., 1999).

These data demonstrated that expression of EGFP-tagged caldesmon at high level led to disruption of stress fibres and focal adhesions in smooth muscle cells, although less severe than that observed in fibroblasts (Helfman et al., 1999). This

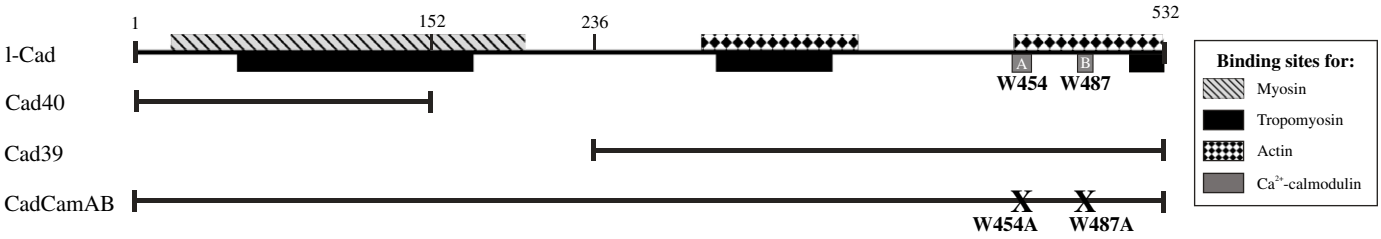


Fig. 2. Schematic diagram of rat l-caldesmon constructs used in this study. The N-terminal fragment of l-caldesmon (Cad40; amino acids 1-152) harbours the myosin-binding site (hatched) (Velaz et al., 1990; Bogatcheva et al., 1993; Redwood and Marston, 1993; Huber et al., 1995) and a tropomyosin-binding site (black) (Smith et al., 1987; Redwood and Marston, 1993; Redwood et al., 1993). The C-terminal fragment of l-caldesmon (Cad39; amino acids 236-532) contains two tropomyosin-binding sites, two actin-interacting regions (diamonds) (Wang et al., 1997b; Marston et al., 1998), and the two Ca^{2+}/calmodulin binding sites A and B, as indicated (dark-grey boxes) (Wang et al., 1991; Marston et al., 1994; Wang et al., 1996). Both Ca^{2+}/calmodulin-binding sites contain a key tryptophan residue (W454 and W487) that is essential for this interaction (Graether et al., 1997); replacement of these tryptophan residues with alanine (W454A and W487A) generates an l-caldesmon mutant that is unable to interact with Ca^{2+}/calmodulin (CadCamAB).

is probably owing to the inherently stable actin stress fibres in A7r5 smooth muscle cells that are reminiscent of the well-developed contractile apparatus of the differentiated smooth muscle cells. As the majority of the EGFP-l-caldesmon-transfected A7r5 cells retained an intact actin cytoskeleton containing EGFP-l-caldesmon-labelled stress fibres, it provides a convenient model with which to study the dynamics of mobilisation of caldesmon during podosome formation.

Effects of expression of EGFP-caldesmon on PDBu-induced podosome formation in smooth muscle cells

Although overexpression of l-Cad caused disruption of stress fibres and focal adhesions, which is a prelude to podosome-formation in A7r5 cells, l-Cad alone was not able to induce the formation of podosomes. Next, we investigated whether overexpression of l-Cad affects the ability of PDBu to induce podosome formation. Whereas PDBu-treatment for 30 minutes caused 40-50% of A7r5 cells to develop podosomes, similar PDBu treatment induced podosome formation in only 20% of cells overexpressing l-Cad after 48 hours of transfection. Furthermore, overexpression of l-Cad significantly reduced the average number of PDBu-induced podosomes per cell: from 60/cell in non-transfected cells to 40/cell in l-Cad-transfected cells. These data suggest that caldesmon, albeit an integral component of podosomes, contributes to the regulation of the dynamics of podosome formation presumably by dampening the assembly phase and/or promoting the turn-over of podosomes, resulting in a decrease in the half-life and, thus, steady-state population of podosomes per cell. As shown in Fig. 3F-I, the exogenous EGFP-l-caldesmon co-localised with the PDBu-induced podosomes and was positioned either at the middle of the actin columns (as shown in Fig. 3H,I) or throughout the entire actin columns (similar to those seen with endogenous caldesmon in Fig. 1C). We are not clear whether this represents a spatial distribution of caldesmon that may occur during different phases of podosome formation.

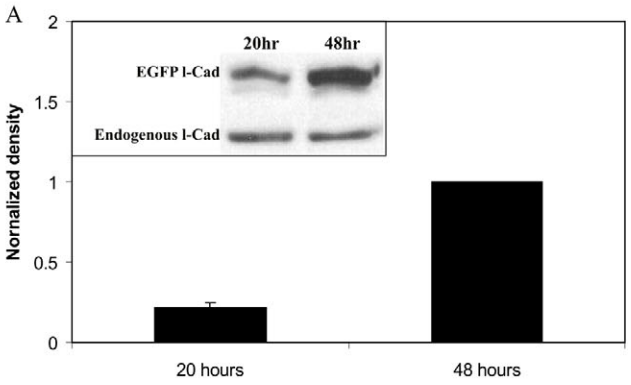
To show the dynamic nature of podosomes and to provide a glimpse of how caldesmon and actin are disassembled from stress fibres and reorganised into podosomes, selected video images of cells co-transfected with EGFP-l-caldesmon and DsRed-cortactin, with and without stimulation with PDBu, are shown in Fig. 4. Without PDBu-treatment (Fig. 4A), EGFP-l-caldesmon localised to stress fibres, while DS Red cortactin was distributed diffusely in the cytoplasm and little change in the cytoskeletal organisation was observed. PDBu treatment caused rapid disruption of stress fibres, and caldesmon became disassembled from stress fibres and reorganised into dynamic cortactin-containing podosomes (Fig. 4B). Podosome-producing cells also underwent very active cytoskeletal reorganisation at the cell periphery, where membrane ruffles containing caldesmon and cortactin were constantly made and turned over (see arrows in Fig. 4B).

siRNA interference of caldesmon expression and podosome formation

To assess further the possible mechanism by which caldesmon may regulate PDBu-induced podosome formation, we employed siRNA interference of caldesmon expression in A7r5 cells. We have generated two siRNAs targeted to mRNA sequences that are identical in rat l-and h-caldesmon, in case knockdown of l-caldesmon may cause compensatory expression of h-caldesmon. The siRNA1120 and siRNA143 both proved to be effective in reduction of caldesmon expression as determined by western blot analyses (Fig. 5A). Based on a transfection efficiency of about 50% for the caldesmon-targeted siRNA, we estimate that on average caldesmon expression was knocked down by 80% and 40%, respectively, by siRNA1120 and siRNA143, normalised to that of the negative control (Fig. 5B).

By inspecting cells transfected with siRNA1120, we first determined how caldesmon knockdown may affect stress fibre organisation without PDBu stimulation. Cells were co-transfected with siRNA1120 and EGFP-β actin, which served to mark the siRNA-transfected cells as well as stress-fibres in the transfected cells. As shown in Fig. 5C, cells exhibiting significant knockdown of caldesmon (~80%) still retained a prominent array of actin stress fibres, which were decorated with the residual caldesmon. These data indicates that a small fraction of normally bound l-caldesmon is sufficient for the formation of actin stress fibres in the A7r5 smooth muscle cells. This is not due to compensatory expression of h-caldesmon in siRNA-transfected cells because siRNA1120 interfered with l- and h-caldesmon expression, and no h-caldesmon was detected in western blots (not shown). As we were unable to achieve total knockdown of caldesmon using any of the siRNAs, it is not clear to what extent the structure of stress fibres would be affected in

caldesmon-null cells. However, stress fibres in the siRNA-transfected cells were less stable as PDBu treatment for 30 minutes caused stress fibre disruption in almost 80% of the siRNA1120-transfected cells compared with 50% in cells transfected with the negative control siRNA.



Next, we determined if caldesmon knockdown affected the ability of PDBu to induce podosome formation. As shown in Fig. 5D,E, knockdown of caldesmon by siRNA1120 enhanced dramatically the ability of PDBu to induce the formation of podosomes. This was verified by measuring the number of podosome per cell and percentage of cells that produced podosomes. PDBu induced over 60% of siRNA1120-transfected cells to produce podosomes with an average of about 90 podosomes per cell. This is compared with 40% of non-transfected or vector control transfected cells, which develop podosomes with an average of 60 podosomes per cell. These findings indicate clearly that underexpression of caldesmon promotes podosome formation and are in agreement with our data showing that overexpression of caldesmon produced the opposite effect by attenuating podosome formation. Our data is also consistent with a previous report that l-caldesmon inhibits in vitro Arp2/3-induced actin nucleation, which plays a crucial role in the assembly of podosomes (Yamakita et al., 2003). Thus,

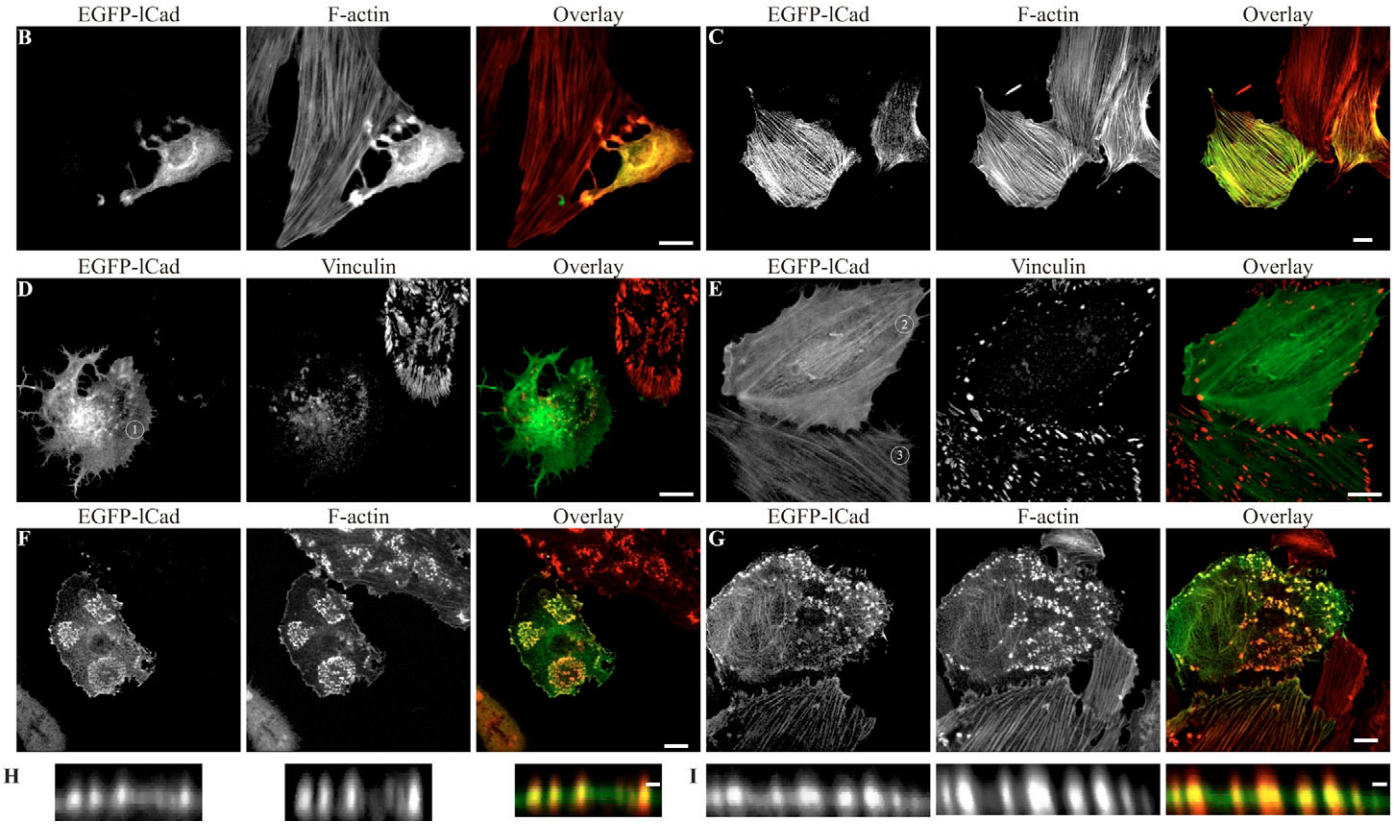


Fig. 3. Overexpression of EGFP/l-caldesmon causes disruption of stress fibres and focal adhesions in smooth muscle cells. (A) A7r5 cells were transfected with a plasmid encoding for EGFP-l-caldesmon for 20 or 48 hours prior to lysis and western blot analysis with a general caldesmon antibody (inset). Densitometry was performed to quantify the level of exogenous EGFP-l-caldesmon expression by normalising the signal to that of endogenous l-Cad. The level of EGFP-l-caldesmon is approximately fivefold higher at 48 hours than at 20 hours. (B-I) A7r5 cells were transfected with a plasmid encoding EGFP/l-caldesmon for 48 hours prior to fixation, and stained using antibodies raised against EGFP (green) and either (B,C,F-I) TRITC-phalloidin (to label F-actin, red) or (D-E) antibodies raised against the focal adhesion protein vinculin (red). (B,D) Expression of high levels of EGFP-l-caldesmon induced the dissolution of stress fibres and focal adhesions (cell 1 in D) in the transfected cells. The untransfected cells contained well-defined stress fibres and large vinculin-stained focal adhesions. (C,E) Moderate to low levels of EGFP-l-caldesmon (cell 2 and 3, respectively, in E) did not cause significant disorganisation of stress fibres but appeared to displace focal adhesions to the cell periphery. (F,G) Confocal micrographs of EGFP-l-caldesmon expressing-cells stimulated with 1 μM PDBu for 30 minutes. Many cells contained large clusters of podosomes, as shown in F, while others had a more scattered distribution of podosomes, as in G. (H,I) Reconstruction of the x-z-profiles of podosomes from cells in F and G showing that EGFP-l-caldesmon co-localised to the actin rich core of the podosome. Bars, 20 μm in B-G; 2 μm in H,I.

caldesmon appears to act as a de-stabilising factor in podosome formation, possibly by inhibiting Arp2/3-mediated actin polymerisation at the core of podosomes. This predicts that expression of caldesmon would be suppressed in invasive cells such as the Src-transformed fibroblasts, which inherently develop prominent podosomes (Tarone et al., 1985). The expression of caldesmon is indeed reduced by 60% compared with that in non-transformed cells (data not shown).

Effects of overexpression of Cad40 and Cad39 in PDBu-induced podosome formation

Next, we determined the requirement of known functional domains of caldesmon in podosome-formation by expressing EGFP-tagged Cad40, the N-terminal fragment containing the myosin-binding sites and Cad39, the C-terminal fragment housing the binding sites for actin, calmodulin and tropomyosin, in A7r5 cells (Fig. 2).

As shown in Fig. 6A, overexpression of Cad40 disrupted stress fibres, forming shorter and thinner actin fibres and sometimes wavy strands in about 50% of the cells. This may be due to binding of Cad40 to endogenous myosin and inhibiting contraction, resulting in the disassembly of stress fibres (Lee et al., 2000; Li et al., 2000). In those Cad40-transfected cells that still retained normal stress fibres, Cad40 did not label stress fibres and appeared as small clusters diffusely distributed in the cytoplasm, confirming that the N-terminal fragment did not interact with actin fibres (Fig. 6B). Cad39, which contained the binding sites for actin, calmodulin and tropomyosin, was less disruptive than Cad40 to the integrity of stress fibres, affecting only 20% of the transfected cells (Fig. 6E). The majority of the Cad39-transfected cells retained prominent stress fibres as shown in Fig. 6F; and, in contrast to Cad40, Cad39 co-localised with actin stress fibres, indicative of its ability to interact with actin, tropomyosin and calmodulin. These results suggest that caldesmon binding to myosin and to actin/tropomyosin/calmodulin contributes to the stability of stress fibres. This stability would be disrupted by overexpressing Cad40 or Cad39, which compete with endogenous caldesmon in interacting with myosin and actin stress fibres, respectively.

PDBu treatment for 30 minutes enhanced the disruption of stress fibres and focal adhesions caused by Cad40 and Cad39, and induced the development of podosomes in many of the transfected cells. As shown in Fig. 6G,H, the exogenous Cad39 co-localised with the PDBu-induced podosomes and were positioned at the core of the actin columns, while Cad40 had a diffuse distribution and was not enriched in podosomes (Fig. 6C,D). These data suggest that the binding sites for actin, calmodulin and tropomyosin at the C-terminal half of caldesmon, but not the myosin-binding sites at the N-terminal end, are required for effective localisation of caldesmon to PDBu-induced podosomes in smooth muscle cells.

Intact calmodulin-binding sites are required for targeting caldesmon to podosomes

We asked if the ability to bind calmodulin is involved in the translocation of caldesmon to podosomes. We generated an EGFP-caldesmon construct (CadCamAB), in which the two tryptophan residues, W454 and W487, known to be essential for calmodulin-binding were mutated to Ala. The W454A and W487A mutations have been shown to abolish interaction between caldesmon and calmodulin, but have little effect on binding of caldesmon to actin (Graether et al., 1997; Wang et al., 1997b; Li et al., 2004). Depending on the expression level, CadCamAB caused various degrees of stress fibre disassembly. Some cells suffered a complete loss of stress fibres characterised by a diffuse distribution of

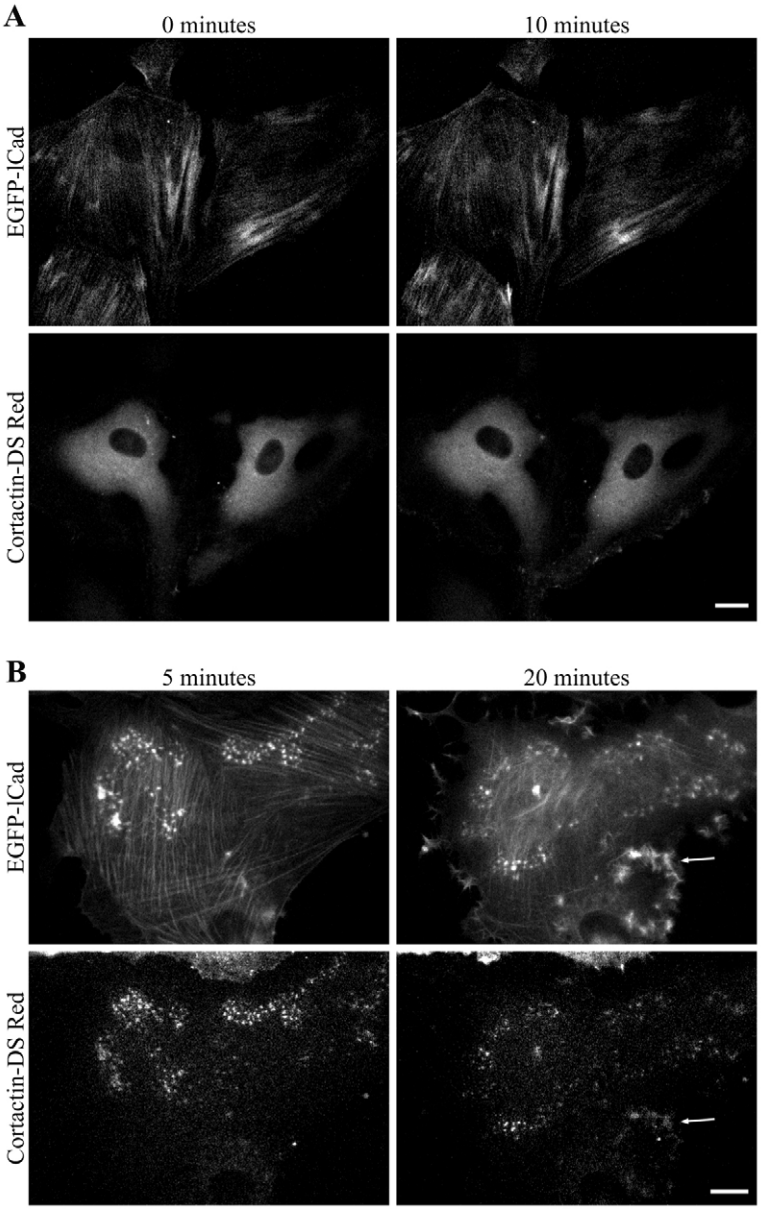


Fig. 4. Dynamic incorporation of EGFP-caldesmon into podosomes in live cells treated with PDBu. Video images of live A7r5 cells expressing EGFP-l-caldesmon and DS Red-cortactin were recorded either in the absence (A) or presence (B) of 1 μM PDBu. The times in A refer to the time from the beginning of the video; the times in B refer to the time after the onset of PDBu stimulation. Bars, 20 μm.

both actin and exogenous CadCamAB (Fig. 7A) and loss of focal adhesions (Fig. 7C). In those CadCamAB-transfected cells where stress fibres still remained intact (Fig. 7B), CadCamAB staining generally aligned with the phalloidin-labelled stress fibres but appeared thicker and more diffuse. PDBu-treatment of CadCamAB-transfected cells significantly augmented disassembly of stress fibres, affecting 80% of the transfected cells (compared with about 50% of cells mock-transfected with EGFP alone), many of which also produced podosomes (Fig. 7D,E). CadCamAB had a diffuse distribution in the cytoplasm and did not co-localise with podosomes (Fig. 7D). X-Z profiles of podosomes showed that CadCamAB was moderately enriched in the immediate areas surrounding the podosomes, but was excluded from the actin core (Fig. 7E).

These results indicate that binding of caldesmon to calmodulin is required for stabilisation of stress fibres, in agreement with Li et al. (Li et al., 2004), who showed that Cad39AB, a Cad39 construct that did not interact with calmodulin, disrupted stress fibres in CHO cells. Our data also show that the integrity of calmodulin-binding sites is required for efficient translocation of caldesmon to podosomes.

Discussion

A number of studies on cell motility have demonstrated a role of caldesmon in the regulation of contractility and stability of the actin cytoskeleton and focal adhesions in non-muscle cells (Kira et al., 1995; Ishikawa et al., 1998; Helfman et al., 1999; Li et al., 2004). Here, we present new evidence that caldesmon is a prominent component of PDBu-induced podosomes in the rat aortic smooth muscle cell line A7r5. In contrast to primary vascular smooth muscle cells, A7r5 cells constitutively express a prominent array of actin stress fibres and focal adhesions (Fig. 1), providing a unique and useful cell model for studying the dynamics of podosome formation involving the

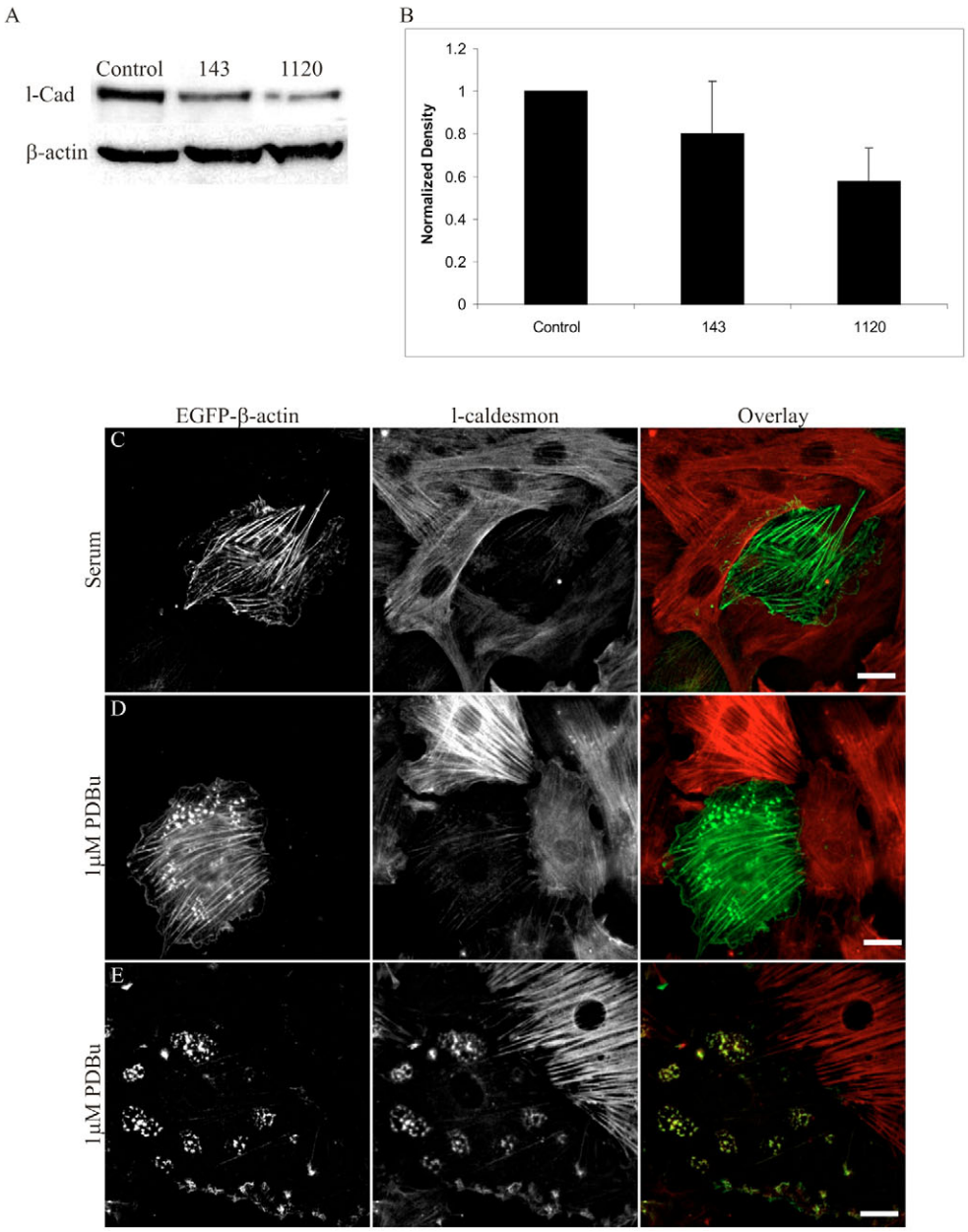


Fig. 5. Knockdown of endogenous l-caldesmon by siRNA enhances podosome formation. (A,B) A7r5 cells were transfected with the caldesmon-specific siRNA1120 (1120) and siRNA143 (143) or with a negative control siRNA (control) for 48 hours prior to lysis and western blot analysis. Samples were probed with a general l-caldesmon antibody (l-Cad) or β-actin as a loading control. (A) Blot is representative of three independent experiments. (B) Densitometry was performed to quantify the level of knockdown by each caldesmon siRNA compared with the negative siRNA control, as described in the Materials and Methods. The caldesmon siRNA143 and siRNA1120 reduced the level of caldesmon in the total population of transfected and un-transfected cells by ~20% and 40%, respectively. This translates to about 40% and 80% knockdown of caldesmon expression, respectively, by siRNA143 and siRNA1120, taking into account of 50% transfection efficiency. (C-E) A7r5 cells co-transfected with siRNA1120 and EGFP-β-actin were fixed and stained 48 hours after transfection (C) or stimulated with 1 μM PDBu for 30 minutes prior to fixation (D,E). Cells were stained for EGFP (green) to identify siRNA transfected cells and l-caldesmon (red). An overlay of the two channels is shown. (C) Cells transfected with siRNA1120 show a significant decrease in the level of caldesmon but still retain stress fibres. (D,E) Stimulation of cells in which the level of caldesmon has been reduced by siRNA1120 with PDBu show an increase in both the number of cells displaying podosomes and the number of podosomes per cell. Bars, 20 μm in C-E.

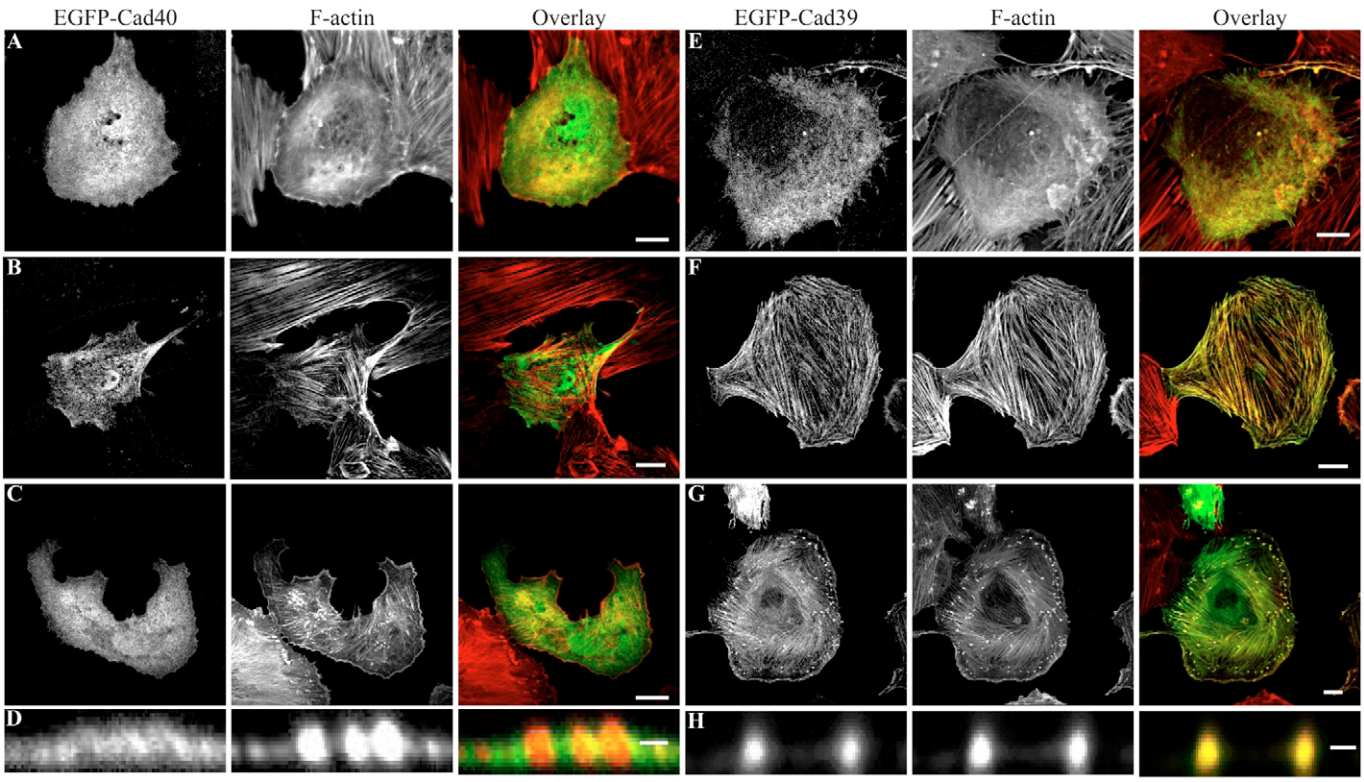


Fig. 6. Cad39 but not Cad40 localises to podosomes. A7r5 cells expressing EGFP-Cad40 (A-D) or EGFP-Cad39 (E-H) fragments of caldesmon were fixed and stained 48 hours after transfection (A,B,E,F) or stimulated with 1 μM PDBu for 30 minutes prior to fixation (C,G). Cells were stained for EGFP (green) or F-actin (red) and an overlay of the two channels is shown. (A,B) Cad40 disrupted stress fibres in 50% of transfected cells (A), while cells expressing lower levels of Cad40 retained stress fibres (B), which did not contain Cad40. (C,D) Cad40 did not translocate to podosomes induced by PDBu. (D) A reconstruction of the x-z profiles of podosomes from cells in C. (E,F) Cad39 was less disruptive to stress fibres, affecting only 20% of the transfected cells (E) and the majority (80%) of the cells showed little disorganisation of stress fibres (F). Unlike Cad40, Cad39 localised to stress fibres. (G,H) Cad39 co-localised to the core of the podosome. (H) The x-z profiles of podosomes from cells in G. Bars, 20 μm in A-C,E-G; 2 μm in D,H.

disassembly of stress fibres (Fultz et al., 2000; Hai et al., 2002; Gimona et al., 2003).

The role of caldesmon in the stability of stress fibres and focal adhesions

Although the detailed mechanisms by which caldesmon contributes to the regulation of cell motility and cell invasion has not been clearly defined, there is evidence to suggest that caldesmon: (1) regulates contractility by modulating the actin-activated myosin ATPase activity (Marston and Redwood, 1993; Dabrowska et al., 1996; Chalovich et al., 1998), and (2) stabilises actin stress fibres by inhibiting actin-severing proteins (Ishikawa et al., 1989a; Ishikawa et al., 1989b; Gusev et al., 1994; Dabrowska et al., 1996). Contractility and stress fibre stability are intimately interdependent of each other, such that any mechanism or reagent that inhibits contractility would also perturb stress fibre stability and vice versa (Chrzanowska-Wodnicka and Burridge, 1996; Choquet et al., 1997; Hirose et al., 1998; Rottner et al., 1999; Riveline et al., 2001; Balaban et al., 2001; Tsubouchi et al., 2002; Burgstaller and Gimona, 2004). Ca^{2+} signals transmitted via Ca^{2+}-binding proteins such as calmodulin are the prime regulator of contraction, acting at either the thick and/or thin filaments by activating myosin light chain kinase and/or releasing caldesmon inhibition of contraction, respectively. Thus, in the presence of Ca^{2+}, calmodulin-bound caldesmon occupies an ‘on’ site on the actin-tropomyosin filament that promotes contraction and stabilises stress fibres. In the absence of Ca^{2+}, calmodulin remains associated with caldesmon but switches caldesmon to an ‘off’ position on the actin thin filament that inhibits contraction and the generation of tension, resulting in destabilising the stress fibres.

This model is consistent with our observation that overexpression of the caldesmon fragments Cad40 or Cad39 in A7r5 cells disrupted stress fibres. It is likely that Cad40, when expressed in excessive amounts, destabilises stress fibres and contraction by competing with endogenous caldesmon for myosin, rendering it inaccessible to stress fibres. Similarly, Cad39 may compete with endogenous caldesmon for actin-tropomyosin filaments and produce non-contractile stress fibres. Thus, overexpression of Cad40 or Cad39 would compromise contraction that eventually led to disruption of stress fibres and focal adhesions. This is consistent with the results that expression of the caldesmon fragments at lower levels did not cause significant disorganisation of stress fibres or focal adhesions.

We have shown before that Trp454 and Trp487, at calmodulin-binding sites A and B, respectively, are essential for caldesmon to interact with calmodulin; mutation of these Trp residues to Ala abolished the caldesmon-calmodulin

interaction (Graether et al., 1997). Overexpression of the full-length l-caldesmon containing the W454A and W487A mutations (CadCamAB) disrupted the normally straight and parallel array of stress fibres as well as focal adhesions. The stress fibres became fragmented and disorganised, while CadCamAB itself became diffuse throughout the cytoplasm (Fig. 7A). Our data agree with a previous report by Li et al. (Li et al., 2004), who showed that forced expression of a Cad39 fragment harbouring the W454A and W487A mutations disrupted the organisation of stress fibres and focal adhesions in CHO cells. These complementary results suggest that the exogenous calmodulin-free CadCamAB displaces the endogenous caldesmon from stress fibres, which became locked in an 'off' conformation that inhibits contraction and tension generation, resulting in disassembly of the stress fibres and focal adhesions. In addition, excess CadCamAB would probably compete with endogenous caldesmon for myosin and further adversely affects contractility and destabilises stress fibres.

Our observation that overexpression of the EGFP-tagged wild-type caldesmon also disrupted stress fibres and focal adhesions corroborates a previous report by Helfman et al. (Helfman et al., 1999), who demonstrated that overexpression of EGFP-caldesmon inhibited non-muscle cell contractility and interfered with the formation of focal adhesions and stress fibres. The degree of disruption appeared to be much less in smooth muscle cells than in fibroblasts, apparently owing to the inherently more stable and well-organised array of actin stress fibres in the A7r5 cells. However, it is not immediately obvious how exogenous full-length caldesmon disrupts stress fibres and focal adhesions, as the exogenous caldesmon can be fully regulated by Ca^{2+}/calmodulin and therefore is expected to behave like the endogenous protein in stabilising the stress fibres. One possibility is that excessive amounts of exogenous caldesmon may compete for the limited quantity of available free calmodulin (Hulvershorn et al., 2001) and interfere with contraction, resulting in disruption of stress fibres and focal adhesion. This can partly explain why Cad39, which retains the calmodulin-binding sites and presumably can compete with endogenous caldesmon for calmodulin, also disrupts stress fibres, although to a lesser extent than full-length caldesmon. However, CadCamAB, which is unable to bind calmodulin but retains an intact myosin-binding site, also disrupts stress fibres, presumably by competing with endogenous caldesmon for myosin.

siRNA interference data showed that significant knockdown of caldesmon expression did not affect the overall structure of stress fibres in the A7r5 cells, which tended to be more spread out and rounded (Fig. 5C). Stress fibres in caldesmon-knockdown cells,

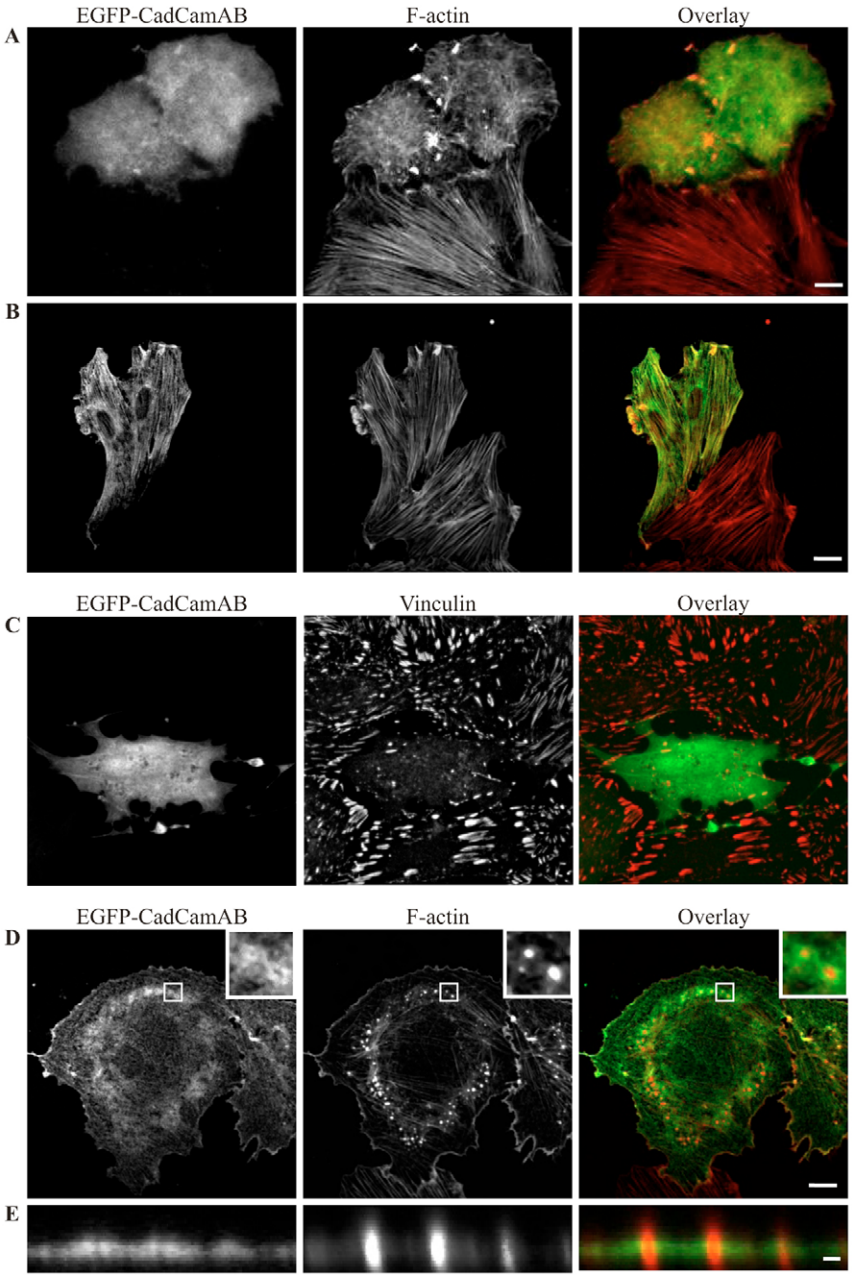


Fig. 7. Interaction with Ca^{2+}/calmodulin is required for the translocation of l-caldesmon to podosomes. A7r5 cells transfected with plasmids encoding EGFP-CadCamAB (green) were either stained with TRITC-phalloidin to identify F-actin (red) (A,B,D,E) or with a monoclonal antibody raised against vinculin (red) to identify focal adhesions (C). The two channels were merged (overlay) to show co-localisation.
(A-C) Expression of EGFP-CadCamAB caused severe disruption of actin stress fibres (A) and focal adhesions (C) in some cells; others retain an intact array of stress fibres (B). (D) Confocal micrograph of CadCamAB-transfected cells stimulated with 1 μM PDBu for 30 minutes prior to staining. Insets in D show a 10×10 μm enlargement of the cell to show the exclusion of CadCamAB from the actin core of podosomes. (E) Reconstruction of the x-z profiles of selected podosomes of the cell in D. Bars, 20 μm in A-D; 2 μm in E.

however, appeared to be less stable and were more prone to disruption by PDBu-treatment. These data have interesting implications: at least in smooth muscle cells, a sub-stoichiometric amount of bound caldesmon appears to be sufficient for stress fibre formation, possibly a result of reduced inhibition of contraction and, thus, enhanced stabilisation of stress fibres.

Taken together, these results suggest that caldesmon plays a role in the regulation of cytoskeletal organisation by interacting with multiple binding partners, including myosin, actin, calmodulin and tropomyosin, which dictates its localisation in different cytoskeletal organelles, such as podosomes and lamellipodia, and subsequent regulation of their formation and function in cell motility and invasion.

Caldesmon is an integral component of PDBu-induced podosomes

The formation of podosomes involves the disassembly of the stress fibres and focal adhesion complexes, and reorganisation of actin with the help of proteins involved in the actin-polymerisation machinery, including cortactin, Arp2/3 and N-WASp (Linder and Aepfelbacher, 2003; Buccione et al., 2004; Linder and Kopp, 2005; Zhou et al., 2005) to form actin column at the core of the podosome. We have recently shown that p21-activated kinase 1 (PAK1) induced the formation of podosomes that contained caldesmon (Webb et al., 2005). Our finding is consistent with an early report demonstrating that caldesmon is present in podosomes in Src-transformed non-muscle cells (Tanaka et al., 1993). Here, we show that PDBu induces disassembly of caldesmon-labelled stress fibres, and the dissociated actin and caldesmon are recruited to the sites of podosome-formation and reorganised into the actin column. We have further identified functional domains required for localisation of caldesmon to podosomes using EGFP-tagged full-length caldesmon, and functional fragments containing binding sites for actin/calmodulin/tropomyosin and myosin II, Cad39 and Cad40, respectively. We show that Cad39 was translocated to the actin core of podosomes, suggesting that binding to actin and calmodulin, rather than to tropomyosin, facilitates translocation of caldesmon to podosomes, as it has been shown before that tropomyosin and myosin II are not present in the actin cores, but appeared as diffuse bands at the periphery of podosomes upon stimulation with PDBu (Burgstaller and Gimona, 2004). This is also consistent with our finding that Cad40, containing the myosin II-binding site and a second tropomyosin-binding site, was excluded from the actin core of the podosomes and appeared as diffuse bands around the podosomes, presumably by virtue of its interaction with myosin II and tropomyosin (Fig. 6C). We conclude that caldesmon is localised to the actin column at the podosomes as a result of its interaction with actin and/or calmodulin, but not with tropomyosin or myosin, which appeared to be located on the outside of the actin columns.

The integrity of the calmodulin-binding sites is essential for localisation of caldesmon to the actin-core of podosomes

PDBu treatment augmented the disruption of stress fibres in CadCamAB-transfected cells and induced podosome-formation in many of the disrupted cells. In contrast to wild-type caldesmon, which localised at the actin core of podosomes (Fig. 3F,G), CadCamAB was excluded from the actin cores but was enriched in areas surrounding the podosomes (Fig. 7D,E). This observation indicates that calmodulin-free CadCamAB was not recruited to the actin columns in podosomes and that calmodulin binding to caldesmon may play a crucial role in podosome formation in vivo, implicating a regulatory role for Ca^{2+} in this process. Our finding is in keeping with the model proposed by Li et al. (Li et al., 2004) that caldesmon in the absence of Ca^{2+}/calmodulin prevents actin bundling. Caldesmon binding to Ca^{2+}/calmodulin allows the binding of fascin, which promotes actin bundling, and the formation of stress fibres (Ishikawa et al., 1998). These results suggest that assembly and stability of the vertical actin fibres at the core of podosomes may be regulated by Ca^{2+}/calmodulin and caldesmon, whereas actin-binding proteins such as Arp2/3, N-WASp and cortactin regulate actin branching from the core.

Caldesmon regulates the dynamics of podosome assembly

Podosomes are dynamic cytoskeletal organelles with a life time of 2-10 minutes that undergo rapid assembly and turnover of actin filaments at the core, which must be tightly regulated. Here, we have provided evidence that caldesmon may regulate podosome assembly by studying the effects of over- and underexpressing caldesmon on podosome formation in A7r5 cells. Overexpression of caldesmon reduces the ability of PDBu to induce podosome formation and, by contrast, siRNA interference of caldesmon expression augments podosome formation. These results suggest that caldesmon regulates podosome formation by either inhibiting the assembly and/or enhancing the turn-over of podosomes. This is consistent with the recent data by Yamakita et al. who showed that caldesmon inhibits Arp2/3-mediated actin nucleation in vitro and this inhibition can be released by either Ca^{2+}/calmodulin or phosphorylation of caldesmon by Cdc2 kinase (Yamakita et al., 2003). As activation of Arp2/3 and NWasp at sites of podosome formation has been shown to be a key step in podosome assembly, it is conceivable that caldesmon may act as an inhibitor of Arp2/3-mediated actin nucleation during podosome assembly. Our data, showing that preservation of the calmodulin-binding sites on caldesmon is a prerequisite for caldesmon translocation to podosomes, further support the finding by Yamakita et al. that interaction between Ca^{2+}/caldmodulin and caldesmon may play a role in the caldesmon-mediated regulation of podosome assembly.

In conclusion, we have provided new evidence that caldesmon is a prominent component of the actin core of PDBu-induced podosomes in smooth muscle cells. Translocation of caldesmon to podosomes requires its ability to interact with actin and calmodulin, suggesting a Ca^{2+}-dependent regulatory mechanism via caldesmon-calmodulin interaction in podosome formation. siRNA interference data implicates a regulatory role for caldesmon in the dynamics of podosome formation, possibly by inhibiting the assembly phase of podosome formation.

Materials and Methods

DNA constructs

pEGFP l-caldesmon was generated by amplifying the *Rattus norvegicus* l-caldesmon (NM_013146) cDNA by PCR using the primers 5′-CTC GAG TAA TGC

TTA GCA GAT CCG GGT CCC AG-3′ (sense) and 5′-CCT AGG TCA GAC CTT AGT GGG AGA AGT GAC-3′ (antisense). The N-terminal fragment of caldesmon (Cad40) was amplified using the same sense primer and 5′-CCA TGG TCA CCG TCC TGA GCG ACT CTC TCC-3′ (antisense). The C-terminal fragment of caldesmon (Cad39) was amplified using sense primer 5′-CTC GAG AAT GAC CCA CAA ACT TAA ACA AAC TGA G-3′ and antisense primer 5′-CCA TGG TCA GAC CTT AGT GGG AGA AGT GAC-3′. The PCR products were ligated into the pCR2.1 TA vector (Invitrogen) prior to subcloning into either the *Xho*I and the *Bam*HI sites (l-caldesmon) or the *Xho*I and *Kpn*I sites (Cad39 and Cad40) of a pEGFP-C2 vector (Invitrogen). Site-directed mutagenesis of the two essential tryptophan residues in calmodulin-binding sites A (W454) and B (W487) to alanine was performed using the Stratagene Quickchange II XL site-directed mutagenesis kit (Stratagene) with primers 5′-CGC AAT ATC AAG AGC ATG GCG GAG AAA GGG AGT GTG T-3′ (sense) and 5′-ACA CAC TCC CTT TCT CCG CCA TGC TCT TGA TAT TGC-3′ (antisense) for site A and primers 5′-CAG CCG CAT CAA TGA AGC GCT AAC TAA ATC GCC GG-3′ (sense) and 5′-CCG GCG ATT TAG TTA GCG CTT CAT TGA TGC GGC TG-3′ (antisense) for site B. Cortactin was amplified from a plasmid encoding full-length *Mus musculus* cortactin cDNA (NM_007803) using primers 5′-CCC TCG AGA GGC CAC CAT GTG GAA AGC CTC TGC AGG CCA TGC-3′ (sense) and 5′-CCG GAT CCG CCT GCC GCA GCT CCA CAT AGT TGG-3′ (antisense). The PCR product was incorporated into the *Xho*I and *Bam*HI sites of the pDsRed N1 vector (Clonetech). All constructs were confirmed by dideoxynucleotide sequencing.

siRNA interference

Two siRNA sequences with 3′dTdT overhang targeting *Rattus norvegicus* l-caldesmon cDNA were generated. The first siRNA targets nucleotides 1120-1138 (siRNA1120) of sequence 5′(GUG CUU CAC UCC UAA AGG C)d(TT); the second siRNA targets nucleotides 143-161 (siRNA143) of sequence 5′(GAG GAA GAA UCC UUG GGA C)d(TT) of l-caldesmon. The siRNAs were synthesised and duplexed with their corresponding complementary strands (Qiagen-Xeragon). Negative control 1 FAM-labelled siRNA, which does not correspond to any sequence in the human, rat or mouse genomes, was purchased from Ambion (Austin, TX). For immunofluorescent analysis, the siRNA (56 nM) and pEGFP-β-actin (BD Bioscience) were co-transfected into A7r5 cells using Lipofectamine PLUS reagent (Invitrogen), as per the manufacturer's recommendations. Forty-eight hours after transfection, cells were processed for immunofluorescent staining as previously described (Webb et al., 2005). Western blot analyses were carried out to assess the efficiency of the siRNA knockdown of cortactin. Briefly, A7r5 cells, seeded at a density of 40,000 cells per well of a 24-well plate, were transfected with siRNA (56 nM) or negative control siRNA (56 nM) using Lipofectamine PLUS reagent (Invitrogen). Seventy-two hours after transfection, cells were lysed by boiling in SDS-sample buffer. Rat aortic tissue was kindly provided by Dr Don Maurice (Queen's University). Samples were separated by SDS-PAGE and transferred to a PDVF membrane. Immunoblot analysis was performed using mouse anti-l-caldesmon (BD Biosciences, Lexington, KY) at a 1:1000 dilution and mouse anti-β-actin antibody (Sigma-Aldrich) at a 1:4000 dilution, a horseradish-peroxidase-conjugated secondary antibody, and enhanced chemiluminescence (Perkin Elmer). A digital image of the film was obtained by use of a desk top scanner. Densitometry was performed on the digital images using Corel Draw and Adobe Photoshop software. The amount of l-caldesmon expressed in control and knockdown cells was normalised to the level of β-actin to control for errors associated with the gel loading and cell lysis procedures. The level of knockdown in siRNA1120 and siRNA 143 transfected cells was reported as a percentage of the control siRNA with the mean (±s.d.) reported. Each knockdown was performed in triplicate in each experiment and is representative of at least three individual experiments.

Cell culture, transfection, stimulation and immunofluorescence microscopy

A7r5 rat smooth muscle cells (ATCC) were grown in low glucose (1 g/l) Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% foetal bovine serum and penicillin/streptomycin (Invitrogen). Cells were plated at a density of 2×10^4 cells per 12 mm diameter glass coverslip for fixed cell imaging or per ΔT dish (Bioptechs) for live cell imaging. Coverslips and ΔT dishes were pre-coated with 10 μg/ml fibronectin (Roche Applied Science). Sixteen hours after plating, cells were transfected using Lipofectamine PLUS reagent (Invitrogen), as per the manufacturer's recommendations, using 0.4 μg of DNA. Podosome formation was induced by the addition of 1 μM phorbol-12,13-dibutyrate (PDBu; Sigma-Aldrich), as previously described (Hai et al., 2002; Gimona et al., 2003).

Cell processing for immunofluorescent imaging and cell imaging was performed as described previously (Webb et al., 2005). The primary antibodies used were: rabbit anti-GFP antibody (Chemicon) at 1:200 dilution; mouse anti-l-caldesmon antibody (BD Biosciences) at 1:300 dilution; and mouse anti-vinculin antibody (clone hVIN-1; Sigma-Aldrich) at 1:200 dilution. Goat anti-rabbit and goat anti-mouse Alexa488-conjugated secondary antibodies (Invitrogen) were used at 1:500 dilution. F-actin filaments were stained using tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma-Aldrich) at 1:500 dilution.

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