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Phosphorylation of AFAP-110 affects podosome lifespan in A7r5 cells

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Summary

AFAP-110 is an actin-binding and -crosslinking protein that is enriched in Src and phorbol ester (PE)-induced podosomes. In vascular smooth muscle cells endogenous AFAP-110 localized to actin stress fibers and, in response to treatment with phorbol-12,13-dibutyrate (PDBu), to actin-rich podosomes. Since PEs can activate PKCα, AFAP-110 is a substrate of PKCα and PKCα–AFAP-110 interactions direct podosome formation, we sought to identify a PE-induced phosphorylation site in AFAP-110 and determine whether phosphorylation is linked to the formation of podosomes. Mutational analysis revealed Ser277 of AFAP-110 to be phosphorylated in PE-treated cells. The use of a newly generated, phospho-specific antibody directed against phosphorylated Ser277 revealed that PKCα activation is associated with PE-induced AFAP-110 phosphorylation. In PDBu-treated A7r5 rat vascular smooth muscle cells, immunolabeling using the phospho-specific antibody showed that phospho-AFAP-110 is primarily associated with actin in podosomes. Although mutation of Ser at position 277 to Ala (AFAP-110^{S277A}) did not alter the ability of AFAP-110 to localize to podosomes, overexpression of AFAP-110^{S277A} in treated and untreated A7r5 cells resulted in an increased number of cells that display podosomes. Video microscopy demonstrated that AFAP-110^{S277A} expression correlates with an increased number of long-lived podosomes. Therefore, we hypothesize that AFAP-110 phosphorylation and/or dephosphorylation is involved in the regulation of podosome stability and lifespan.

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Key words: AFAP1, AFAP-110, Podosomes, PKC, Invadopodia, Actin filaments

Introduction

Podosomes are dynamic actin-rich adhesion structures that have been identified in highly motile and invasive cells, such as macrophages, osteoclasts, dendritic cells and transformed fibroblasts (Marchisio et al., 1984; Tarone et al., 1985; Linder et al., 1999; Burns et al., 2001). Podosome formation can also be induced by phorbol ester (PE) treatment in other cell types, such as vascular smooth muscle and endothelial cells (Fultz et al., 2000; Moreau et al., 2003). Morphologically podosomes display an F-actin-rich core that is surrounded by a ring structure containing integrins and integrin-associated proteins. The integrins are distributed in an isotype-specific manner with β1 integrin localizing to the core and β2 integrin and β3 integrin localizing to the ring structure (Linder and Aepfelbacher, 2003). A variety of other molecules have been detected in podosomes, including cytoskeleton-associated proteins, Tyr kinases, Ser/Thr kinases, integrins and RhoGTPases. With the identification of podosome-associated proteins much progress has been made on determining mechanisms responsible for podosome formation. One of the first observations that had been established was that the integrity of microtubules and the actin cytoskeleton is crucial for podosome formation, because microtubule-depolymerization by nocodazole treatment and F-actin-disassembly with cytochalasin D both lead to a loss of podosomes (Lehto et al., 1982; Linder et al., 2000). Further, F-actin in the podosome core has a high turnover rate (Destaing et al., 2003), indicating continuous actin polymerization that involves cortactin, Wiskott-Aldrich syndrome protein (WASP) and Arp2/3. In fact, blocking the functions of cortactin, N-WASP and the Arp2/3 complex using dominant-negative mutants and RNA interference inhibits podosome formation (Linder et al., 1999; Mizutani et al., 2002; Kaverina et al., 2003; Tehrani et al., 2006; Webb et al., 2006). In vivo, macrophages from Wiscott-Aldrich-syndrome patients, who express a mutated WASP protein, are unable to assemble podosomes (Linder et al., 1999). The importance of not only regulating actin polymerization but also depolymerization in the podosome core is evident by the influence of gelsolin, an actin-severing protein that is indispensable for podosome assembly in osteoclasts (Chellaiah et al., 2000). Consistently, overexpression of the gelsolin antagonist caldesmon, which stabilizes actin filaments against actin-severing proteins, also abolishes podosome formation (Eves et al., 2006; Morita et al., 2007). In addition, Erk-dependent phosphorylation of caldesmon is known to weaken its interaction with actin (Foster et al., 2004) and makes F-actin accessible to severing-proteins such as gelsolin. Therefore, polymerization and depolymerization of F-actin and its associated proteins appear to have a crucial role in podosome dynamics. Hence, modifications to actin-associated proteins that alter their actin-binding and actin-organization properties are important mechanisms of podosome regulation.

However, it is noteworthy that F-actin is also crosslinked within podosomes, and less is known regarding the role of actin-filament-crosslinking proteins in podosome formation.

Actin filament-associated protein 1 (AFAP1; also known and hereafter referred to as actin-filament-associated protein of 110 kDa, AFAP-110) belongs to the functional group of actin-binding proteins that regulate actin-filament crosslinking through its ability to undergo dynamic changes in multimerization (Qian et al., 2004). AFAP-110 is composed of a functional actin-binding domain (Qian et al., 2000) and several protein-binding motifs, including two SH2 (Guappone et al., 1998) and one SH3 (Guappone and Flynn, 1997) binding motifs, two pleckstrin homology (PH) domains and a leucine-zipper (LZ) motif (Qian et al., 1998; Qian et al., 2004). In quiescent cells AFAP-110 colocalizes to actin stress filaments (Flynn et al., 1993). Upon knockdown of AFAP-110 using small hairpin RNA (shRNA), actin stress filaments are lost, cell adhesion to fibronectin is reduced 2.7 times and cells fail to form focal adhesions (Dorfleutner et al., 2007). Therefore, the actin crosslinking function of AFAP-110 is involved in the formation of actin stress fibers, which is crucial for regulating cell contractility and cell adhesion. The SH3-binding motif of AFAP-110 has been shown to interact with the SH3 domains of the Tyr kinases Src, Fyn and Lyn. This SH3 interaction is necessary and sufficient to direct Src activation and results in phosphorylation of AFAP-110 on Tyr residues (Reynolds et al., 1989; Flynn et al., 1993; Baisden et al., 2001a; Gatesman et al., 2004). Mutation of the proline residue at position 71 to Ala (P71A) within the SH3-binding motif abolishes Src binding to AFAP-110 and prevents AFAP-110 from directing Src activation. The N-terminal PH domain (PH1) was found to bind to the protein kinase C (PKC) α, β, γ and λ isoforms and, upon PKCα activation, AFAP-110 subsequently becomes phosphorylated on Ser/Thr residues (Qian et al., 2002; Qian et al., 2004). Phosphorylation of AFAP-110 will induce a conformational change that releases autoinhibitory intramolecular interactions and correlates with an acquired ability to activate Src, alter actin filament crosslinking and induce podosome formation (Gatesman et al., 2004). Thus, AFAP-110 might have an important role in podosome formation as an actin-filament-crosslinking protein and as an adaptor protein that relays PKCα signals that direct Src activation.

As AFAP-110 is phosphorylated on Ser/Thr residues in response to PKCα activation and PE treatment, and phosphorylation of AFAP-110 on Ser/Thr residues correlates with a change in structure and function, we sought to identify a site for Ser/Thr phosphorylation and evaluate its specific role in AFAP-110 function. We investigated whether PE-induced AFAP-110 phosphorylation occurs concomitant with podosome formation in vascular smooth muscle cells. PEs are well-known activators of classical (α, β, γ) and novel (δ, ε, η and θ) PKC isoforms. To date, PKCα and δ have been found to participate in podosome regulation (Hai et al., 2002; Tatin et al., 2006). Our data indicate that PE-induced PKCα activation leads to phosphorylation of Ser277 in AFAP-110. Mutation of Ser277 to Ala did not affect localization to podosomes, but did result in an increased number of cells with podosomes in A7r5 cells, which correlated with an increase number of longer-lived podosomes.

Results

Endogenous AFAP-110 localizes to PE-induced podosomes in A7r5 rat smooth muscle cells

To address localization of endogenous AFAP-110 in response to PE stimulation and subsequent podosome formation, the rat aortic smooth muscle cell line A7r5 was utilized, a well-characterized cell system for podosome formation (Fultz et al., 2000; Brandt et al., 2002; Hai et al., 2002; Burgstaller and Gimona, 2005; Eves et al., 2006; Zhou et al., 2006). Immunofluorescence staining of uninduced A7r5 smooth muscle cells displayed a well-formed actin cytoskeleton, highlighted by prominent bundled actin stress fibers (Fig. 1A, panel a). AFAP-110 has been previously described to localize to actin stress fibers in fibroblasts, epithelial and endothelial cells. In smooth muscle cells, AFAP-110 also localized to actin stress fibers (Fig. 1A, panel b). Stimulation of A7r5 cells with the PE phorbol-12,13-dibutyrate (PDBu), resulted in dissolution of stress fibers and formation of actin-rich podosomes (Fig. 1A, arrows in

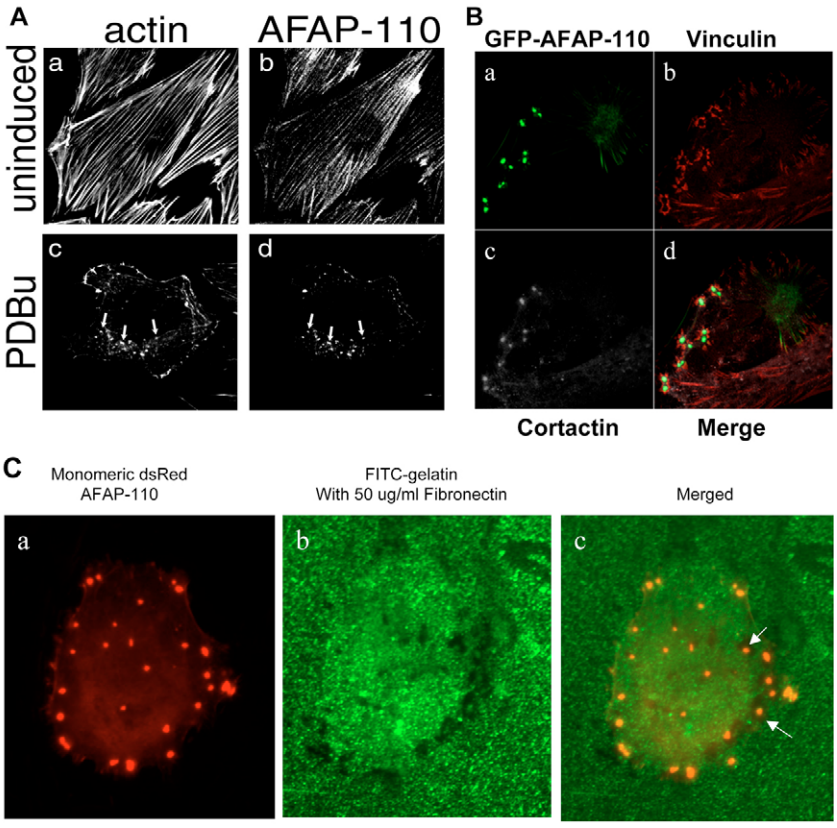

Fig. 1. (A) AFAP-110 localizes to PE-induced podosomes in A7r5 smooth muscle cells. A7r5 cells plated on fibronectin coated glass coverslips were either left untreated (a,b) or were treated with 1 μM PDBu for 1 hour (c,d). Cells were stained with Rhodamine-labeled phalloidin to label F-actin (a,c) and with a polyclonal AFAP-110 antibody (b,d). Confocal microscopy was used to scan 0.7-μm-thick images along the ventral membrane of the cells to image the podosomes. (B) GFP-AFAP-110 was transiently transfected into A7r5 cells and immunofluorescence images were obtained using antibodies to detect endogenous vinculin (b) and cortactin (c), to contrast with GFP fluorescence (a) in the merged image (d). (C) Monomeric dsRed-AFAP-110 was transiently expressed in A7r5 cells that had been plated on an FITC-gelatin matrix containing 50 μg/ml fibronectin.

panel c). Consequently, endogenous AFAP-110 was found to colocalize to actin-rich podosomes (Fig. 1A, arrows in panel d).

These structures were defined as podosomes based on size, shape and localization to the ventral membrane. In cells that ectopically express GFP-AFAP-110, these structures also contained cortactin (a molecular marker for podosomes) and a vinculin ring, which is characteristic of podosomes (Fig. 1B, panels a-d). Identification of these structures as podosomes is also consistent with the observed degradation of an FITC-gelatin matrix beneath these structures (Fig. 1C, panels a-c). Thus, AFAP-110 is associated with actin stress fibers and localizes to PE-induced podosomes in A7r5 smooth muscle cells, which is in agreement with the prediction of previous studies (Qian et al., 2000; Gatesman et al., 2004; Burgstaller and Gimona, 2004).

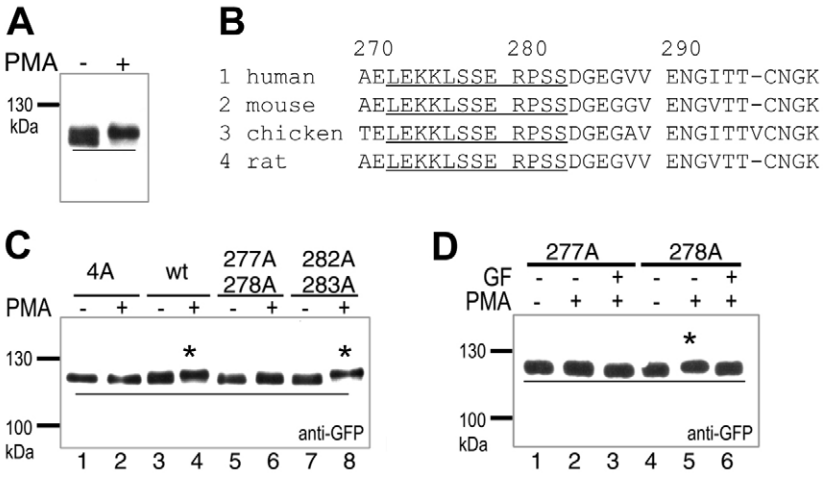

Fig. 2. PE treatment causes a gel shift of the GFP-AFAP-110 protein band in western blot analysis that is dependent upon Ser277. (A) Western blot analysis of GFP-AFAP-110 transfected COS-7 cell lysates. 48 hours post transfection cells were either left untreated (–) or treated for 1 hour with 100 nM PMA (+), directly lysed in SDS sample buffer and subjected to SDS-PAGE and subsequent western blotting with GFP antibody. PMA treatment causes a gel shift of the GFP-AFAP-110 protein band. The horizontal line is shown to contrast the gel shift. (B) Protein sequence alignment of the Ser/Thr rich region between different species. Conserved Ser residues that represent putative PKC target sites – [R/K]XX**[S/T]** and [R/K]X**[S/T]** (Pearson and Kemp, 1991) – and are underlined and have subsequently been mutated to Ala. (C,D) Western blot analysis of GFP-AFAP-110 phosphorylation mutants. (C) Ser277, Ser278, Ser282 and Ser283 (as indicated in 2B) were mutated to Ala to create the 4A mutant (lanes 1 and 2). For double mutants Ser277 and Ser278 or Ser282 and Ser283 were mutated to Ala to create S277A-S278A or S282A-S283A mutants (277A278A and 282A283A lanes 5,6 and 7,8, respectively). (D) For single mutants, Ser277 or Ser278 were mutated to Ala to create S277A or S278A (277A or 278A, respectively). All constructs were transfected into COS-7 cells and 48 hours post transfection treated as indicated with PMA for 1 hour, while inhibition of PKC was accomplished using the PKC inhibitor GF109203X (GF) for 30 minutes. All blots were probed with anti-GFP antibody and the gelshifted protein bands are indicated by an asterisk. Molecular mass markers are indicated on the left. The horizontal line is shown to contrast the gel shift.

PE treatment causes a gel shift of the AFAP-110 protein band

PEs are known to cause activation of classical and novel members of the PKC family of Ser/Thr kinases. AFAP-110 is a binding partner for PKCα, a target of PKCα phosphorylation in vitro and becomes phosphorylated on Ser residues upon PE treatment in vivo (Qian et al., 2002). Here we show that treatment of GFP-AFAP-110-overexpressing cells treated with the PE phorbol-12-myristate-13-acetate (PMA) resulted in a gel shift of the AFAP-110 protein band in western blot analysis (Fig. 2A). Gel shifts during SDS-PAGE can occur when a protein is phosphorylated, which prevents SDS from coating the protein efficiently, resulting in a slightly retarded migration to the cathode and a higher relative mass of the protein. Thus, because PMA is a well-known activator of PKC family members such as PKCα (Kazanietz, 2000), and AFAP-110 is a substrate and binding partner for PKCα (Qian et al., 2002), we predicted that the resulting gel shift was associated with increased AFAP-110 phosphorylation on Ser/Thr residue(s). Previous studies have found that phosphorylation of AFAP-110 by recombinant PKCα can direct a conformational change in AFAP-110 (Qian et al., 2004). Subsequently, we determined that amino acids 270-300 are a major target region for AFAP-110 phosphorylation in vitro (Lidia Cherezova and D.C.F., unpublished data). Sequence comparison of this region among different species revealed highly conserved Ser residues that are consistent with consensus PKC phosphorylation sites (Fig. 2B). To identify the phosphorylated Ser residues, we created a mutant AFAP-110 construct in which all relevant Ser residues (positions 277, 278, 282 and 283 according to the human AFAP-110 sequence) were changed to Ala (hereafter referred to 4A mutant). No gel shift of the A4 mutant protein (isolated from 4A-transfected cells stimulated with PMA) was apparent when compared with AFAP-110 wild-type (Fig. 2C, lanes 1 and 2), which indicated that at least one of the four Ser residues is a potential phosphorylation target. To further characterize the exact phosphorylation site, double mutants of Ser277-Ser278 and Ser282-Ser283 to Ala277-Ala278 and Ala282-Ala283, respectively, were generated and the mutant proteins were tested for the phosphorylation-dependent gel shift after treating the transfected cells with PMA. Fig. 2C shows that the S277A-S278A mutant of AFAP-110 did not result in a gel shift (Fig. 2C, lanes 5 and 6) of the protein band in western blotting, whereas the S282A-S283A mutant protein (Fig. 2C, lanes 7 and 8) still shifted similarly to wild-type AFAP-110 after PMA treatment of transfected cells (lanes 3 and 4). Therefore, the gel shift was attributed to phosphorylation of Ser277 and/or Ser278. Single Ser substitution of these residues revealed that the Ser277Ala mutation prevents the AFAP-110 band gel shift in western blots subsequent to PMA treatment of cells (Fig. 2D, lanes 1 and 2). In addition, we found that pretreatment of cells with the PKC inhibitor bisindolylmaleimide I (GF109203X; Fig. 2D, lanes 5 and 6) will prevent a gel shift of the AFAP-110 protein band after PMA treatment of cells. Previously published observations have indicated that AFAP-110 is phosphorylated on four distinct tryptic fragments in response to treatment with PMA (Qian et al., 2002). Thus, although our data indicate that the presence of Ser277 is required for the gel shift and is a good candidate site for phosphorylation, other sites for phosphorylation do exist in AFAP-110 that might contribute to the gel shift as well. In summary, the observed PMA-induced gel shift could be associated with phosphorylation of AFAP-110 through activation of a PKC pathway and Ser277 is a possible candidate phosphorylation site.

Generation and characterization of a phospho-specific antibody

To define the phosphorylation state of endogenous AFAP-110 and to further study the regulation of AFAP-110 phosphorylation, a

phospho-specific antibody was raised against an 11-amino-acid peptide starting at Leu273 of the human AFAP-110 sequence that included phosphorylated Ser277. In a dot-blot assay non-phosphorylated or phosphorylated peptides were spotted at different concentrations on a nitrocellulose membrane and hybridized with the generated antiserum. The pre-immune serum did not contain antibodies directed against the non-phosphorylated or phosphorylated peptide (Fig. 3A). However, antiserum generated from immunization with the phosphorylated peptide (anti-pSer277) contained antibodies that immunoreacted specifically with the phosphorylated peptide in a dose dependent manner but not with the non-phosphorylated peptide.

To determine whether the gel shift shown in Fig. 2 correlated with AFAP-110 phosphorylation on Ser277, a western blot was performed using the anti-pSer277 antibody. Fig. 3B shows that PMA treatment enabled the anti-pSer277 antibody to recognize AFAP-110; however, this same antibody was unable to recognize GFP-AFAP-110 that had Ser277 mutated to an Ala residue (AFAP-110^{S277A}). The western blot was stripped and reprobed with an antibody against total AFAP-110, which demonstrated equal protein expression levels (Fig. 3B, bottom panel). These data indicate that PMA treatment enables anti-pSer277 antibodies to become immunoreactive with AFAP-110. Thus, we hypothesize that Ser277 is a site for PMA-directed phosphorylation.

To establish the time course of phosphorylation of Ser277, cells were stimulated with PMA and then harvested at the indicated times (Fig. 3C). The anti-pSer277 antibody was immunoreactive with GFP-AFAP-110 subsequent to PMA treatment, and we observed that immunoreactivity to phospho-GFP-AFAP-110 accumulated gradually until maximum immunoreactivity was reached after 1 hour of PMA treatment. At later time points immunoreactivity with anti-pSer277 antibodies declined but was still detectable for up to 24 hours after PMA stimulation (Fig. 3C).

The sequence alignment between human, chicken, rat and mouse AFAP-110 in Fig. 2B shows that Ser277 is conserved between species; therefore, the cross-immunoreactivity of the anti-pSer277 antibody was tested. Fig. 3D shows immunoreactivity with anti-pSer277 antibodies against AFAP-110 from cells overexpressing human and avian AFAP-110, as well as endogenous AFAP-110 immunoprecipitated from in A7r5 rat vascular smooth muscle cells and a human breast cancer cell line that has previously been described to have high levels of AFAP-110 (Dorfleutner et al., 2007). Collectively, these data indicate that avian, human and rodent AFAP-110 become immunoreactive with the anti-pSer277 antibody after PE treatment, and that the newly generated AFAP-110 phospho-specific antibody is highly specific for Ser277-phosphorylated AFAP-110.

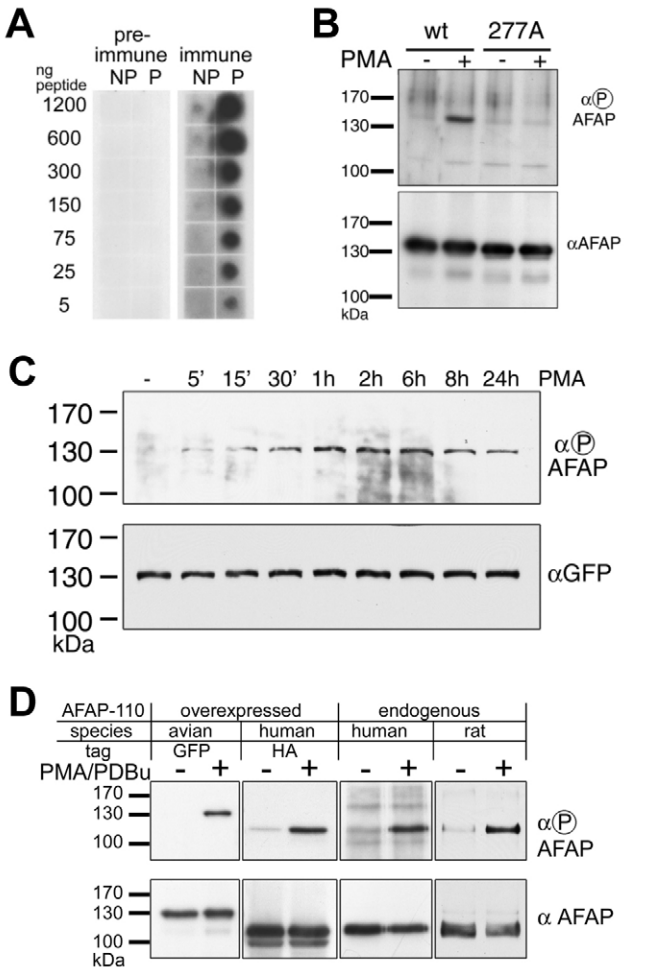

Fig. 3. Characterization of the anti-pSer277 antibody as a potential phospho-specific AFAP-110 antibody. (A) Specificity of the newly generated phospho-specific AFAP-110 antibody was determined by dot-blot assay as described in Materials and Methods. NP, non-phosphorylated peptides; P, phosphorylated peptides. (B) Western blot analysis with the newly generated phospho-specific AFAP-110 antibody anti-pSer277. Samples were prepared as described in Fig. 2A,C, where a gel shift of the AFAP-110 protein band was observed in response to PMA treatment in western blot analysis. Here the western blot was probed with anti-pSer277 phospho-specific AFAP-110 antibody (α(P) AFAP, upper panel) or with an antibody against total AFAP-110 (αAFAP, lower panel) to control for equal expression of AFAP-110. (C) Time course of PMA-induced immunoreactivity with anti-pSer277 antibodies. GFP-AFAP-110 transfected COS-7 cells were treated with 100 nM PMA for the indicated time, prior to lysis in sample buffer, separation by SDS-PAGE and subsequent western blot analysis with anti-pSer277 (α(P) AFAP, upper panel). After stripping the membrane it was re-probed with a GFP antibody (lower panel), to control for equal loading. (D) Anti-pSer277 antibodies are immunoreactive with AFAP-110 in cells treated with PMA/PDBu. Anti-pSer277 antibody (α(P) AFAP, upper panel) recognizes PMA/PDBu-induced phosphorylation of overexpressed avian and human AFAP-110 as well as of endogenous human and rat AFAP-110 in western blot analysis. Overexpression of AFAP-110 was performed in COS-7 cells that had been lysed directly after PMA treatment. Endogenous AFAP-110 was immunoprecipitated after PMA treatment, as indicated, and SDS-PAGE and western blot analysis was performed. Membranes were probed with anti-pSer277 antibodies (α(P) AFAP, upper panel) and with a anti-AFAP antibody (lower panel), to control for equal loading. All western blots are labeled with molecular mass markers on the left.

The presence of the PH1-domain of AFAP-110 is required for AFAP-110 phosphorylation

To characterize the domains important for phosphorylation of Ser277 in AFAP-110 we used mutated AFAP-110 constructs as shown in Fig. 4A. Mutation of Pro71 to Ala will prevent AFAP-110 interaction with SH3 domain-containing binding partners, such as Src (Guappone and Flynn, 1997) and AFAP-110^{P71A} is unable to activate Src in response to PE treatment (Gatesman et al., 2004). Deletion of the first PH domain (ΔPH1) will prevent AFAP-110 interaction with PKCα, β, γ or λ isoforms (Qian et al., 2002). Deletion of the C-terminal 114 amino acids (1-616 mutant) removes the ability of AFAP-110 to bind to actin (Qian et al., 2000) and deletion of the C-terminal 177 aa (GFP-AFAP-110 1-553 mutant) removes the actin-binding domain as well as the LZ motif which has been shown to be important for intra- and intermolecular interactions of AFAP-110 through LZ-LZ-domain or LZ-PH1-

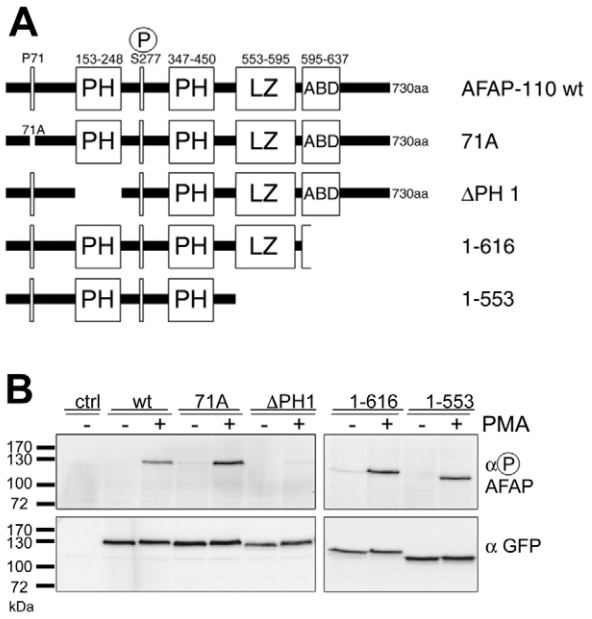

Fig. 4. The PH domain is required for phosphorylation of Ser277. (A) Schematic of mutants used for the mapping of domains that are indispensable for AFAP-110 phosphorylation on Ser277. P71, Pro71 of the SH3-interaction domain; 71A, Pro71 to Ala mutation; PH, pleckstrin homology; LZ, leucine zipper; ABD, actin-binding domain. (B) Western blot analysis of AFAP-110 mutants described in Fig. 4A. 48 hours post transfection cells were treated for 1 hour with 100 nM PMA as indicated, directly lysed in sample buffer and SDS-PAGE and western blot analyses were performed. Western blot results using anti-pSer277 antibody (α(P) AFAP; upper panel), and anti-GFP antibody (lower panel) to control for equal loading. Molecular mass markers on the left of the blots.

domain binding (Qian et al., 2004). Fig. 4B shows that, in response to PMA treatment, SH3 interactions were not necessary for AFAP-110 phosphorylation on Ser277; interestingly, actin-filament binding and oligomerization were also unnecessary. However, if the N-terminal PH1 domain was deleted, AFAP-110 was not immunoreactive with anti-pSer277 after PMA treatment and, thus, predicted not to be phosphorylated on Ser277 in response to PMA treatment. We did note a slight gel shift of GFP-AFAP-110ΔPH1 (Fig. 4B, anti-GFP blot) after exposure to PMA. Thus, because AFAP-110 is known to be phosphorylated on four tryptic fragments in vivo upon exposure to PMA, it might be that another distinct phosphorylation site has been phosphorylated, which led to this slight shift. Nevertheless, this experiment indicates that the presence of the PH1 domain is required for PMA-directed phosphorylation of Ser277.

PKCα phosphorylates AFAP-110 on Ser277 in vivo

AFAP-110 is a substrate for Ser/Thr phosphorylation in response to PKCα activation in vitro and in vivo, and the PH1 domain of AFAP-110 can bind to four different PKC isoforms (α,β,γ,λ), but not the other six isoforms of PKC (Qian et al., 2002). Of the four PKC isoforms that bind to AFAP-110, only α, β and γ are directly activated by PMA (Nishizyka, 1992). Furthermore, in the cells we use, PKCα is expressed at highest levels and we do not detect PKCγ, consistent with the fact that this isoform has been associated with neuronal cells (Saito and Shirai, 2002). Thus, we predicted that PKCα may be responsible for directing phosphorylation of Ser277, either directly or through its ability to activate another Ser/Thr kinase that in turn can phosphorylate AFAP-110 at this residue.

To begin to understand which kinases might be required for phosphorylation of Ser277 in response to PE stimulation, we used the broad-spectrum inhibitor staurosporine (STS) for PKCα and other Ser/Thr kinases. Cells were pretreated with STS before PMA induction. Fig. 5A shows that STS inhibits AFAP-110 phosphorylation in a dose-dependent manner. AFAP-110 has also been shown to interact with and become phosphorylated on Tyr residues by Src Tyr kinase (Guappone and Flynn, 1997), which is activated in a PKC-dependent manner in response to PE treatment of cells (Bruce-Staskal and Bouton, 2001; Brandt et al., 2002). To investigate whether Src activation is a prerequisite of Ser phosphorylation or whether Ser phosphorylation was independent of Src kinase activity and, hence, upstream of PMA-induced Src activation, cells were pretreated with the Src inhibitor PP2 before induction with PMA. PP2 was not able to block PMA-induced phosphorylation of Ser277 (Fig. 5A), indicating Ser phosphorylation of AFAP-110 is upstream of or independent of Src activation. This confirms our results shown in Fig. 4B where mutation of the AFAP-110-binding site for Src (which would prevent AFAP-110 from activating Src in response to PMA stimulation) did not affect PMA-induced AFAP-110 phosphorylation on Ser277. Thus, PE-directed phosphorylation of AFAP-110 on Ser277 occurs upstream or independently of Src activation.

PKC isoforms have an important role in PE-induced podosome formation (Hai et al., 2002; Tatin et al., 2006) and, therefore, their involvement in the Ser phosphorylation of AFAP-110 was tested. Cells were pretreated with the inhibitors Safingol or rottlerin, which can block some PKC family members, as well as other Ser/Thr kinases (Davies et al., 2000). Fig. 5B shows that Safingol, which blocks PKCα, was able to block PMA-induced AFAP-110 phosphorylation – unlike rottlerin, which does not inhibit PKCα (Soltoff, 2007). Overexpression of a myristylated PKCα construct, which serves as a dominant-active form of PKCα, was able to direct phosphorylation of AFAP-110 on Ser277 (Fig. 5C). PMA treatment caused an increase of AFAP-110 phosphorylation in cells expressing myrPKCα, which may be due to activation of endogenous PKCα, resulting in a substantial increase in AFAP-110 phosphorylation. Lastly, treatment of cells with GF109203X, which has reasonably good specificity for PKCα, resulted in a substantial reduction in the phosphorylation of Ser277 (Fig. 5D). It is important to add that these experiments were conducted in COS-7 cells, which contain substantial levels of PKCα. COS-7 cells are also able to form podosomes in response to PE treatment (supplementary material Fig. S1). These data indicate that PKCα can directly phosphorylate Ser277 in AFAP-110.

Phosphorylated AFAP-110 localizes to podosomes

To determine where phosphorylated AFAP-110 is localized, immunofluorescence staining was performed on A7r5 cells that overexpress GFP-AFAP-110. Western blot analysis showed that AFAP-110 was phosphorylated on Ser277 in A7r5 cells (Fig. 3D), and these cells were therefore used for imaging. In untreated A7r5 cells GFP-AFAP-110 localized to actin stress fibers and this localization is consistent with the location of endogenous AFAP-110 (see Fig. 1). There was no signal using the anti-pSer277 antibody (Fig. 6, panels a-d) in unstimulated cells. After PDBu treatment GFP-AFAP-110 and phospho-AFAP-110 localized strongly to podosomes (Fig. 6, panels e-h). However, we were unable to detect endogenous phospho-AFAP-110 using anti-pSer277

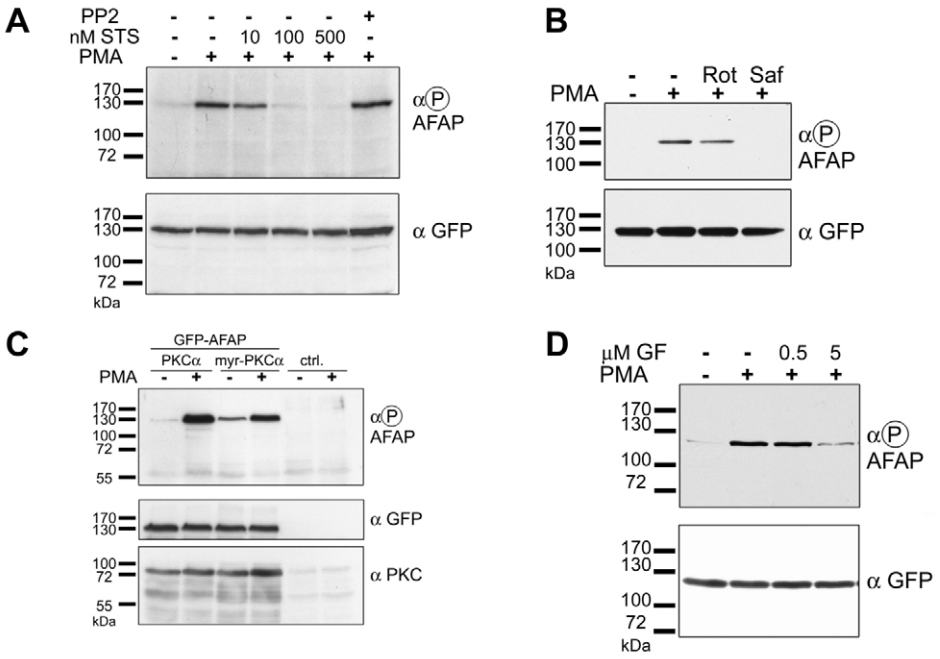

Fig. 5. PKCα is involved in phosphorylation of AFAP-110 on Ser277. (A,B) Kinase inhibitors were added to GFP-AFAP-110 transfected COS-7 cells before PMA treatment for 1 hour. Staurosporine (STS) to inhibit Ser/Thr kinases and PP2 for Src kinase (A), and Safingol (Saf) and rottlerin (Rot) to selectively inhibit certain PKC isoforms as well as other Ser/Thr kinases (B). Cell lysates were separated by SDS-PAGE, and western blotted with antibodies against anti-pSer277 (α(P) AFAP, upper panel), or anti-GFP (lower panel) to control for equal loading. (C) PKCα or the dominant-active myristoylated (myr-) form of PKCα were co-expressed with GFP-AFAP-110 in COS-7 cells and cells were stimulated with 100 nM PMA for 1 hour as indicated. Control cells were left untransfected and also subjected to PMA treatment as indicated. Cells were directly lysed in sample buffer and subjected to SDS-PAGE and western blot analysis using anti-pSer277 (α(P) AFAP; top panel), anti-GFP (α GFP; middle panel) or anti-PKC (α PKC; bottom panel). All blots are labeled with molecular mass markers on the left. (D) Similarly, cells expressing GFP-AFAP-110 were pre-treated with GF109203X (GF), followed by stimulation with 100 nM PMA for 1 hour as indicated. Cells were directly lysed in sample buffer and subjected to SDS-PAGE and western blot analysis using anti-pSer277 (α(P) AFAP, top panel) or anti-GFP (α GFP; bottom panel).

with a good signal-noise ratio in response to PDBu. This might be due to lower expression levels, and only a fraction of endogenous AFAP-110 might be phosphorylated on Ser277, which would yield phospho-AFAP-110 levels at or below detection limits. Therefore, we conclude that GFP-AFAP-110 becomes phosphorylated on Ser277 in A7r5 cells upon PDBu treatment and is localized to podosomes.

Mutation of Ser277 to Ala leads to an increased number of cells with podosomes

We sought to determine whether phosphorylation of Ser277 has a role in PE-directed podosome formation. Upon GFP-AFAP-110 overexpression we noticed three different phenotypes based on AFAP-110 colocalization with actin filaments in A7r5 cells (Fig. 7A). One phenotype displayed stress filaments only, referred to as [A]; a second displayed a mix of stress filaments and podosomes referred to as [A']; and a third showed podosomes only and is referred to as [A"]. The occurrence of these phenotypes was quantified in Fig. 7B. In untreated cells the majority (76%) display AFAP-110 colocalized with stress filaments, and cortactin localized to the cytoplasm. In untreated cells, there was also a less prevalent population of cells (21%) that had a mixture of stress fibers and some small podosomes, indicating some capacity for these cells to spontaneously produce podosomes. Only 3% of untreated cells showed only podosomes with no evidence for well-formed stress filaments. Spontaneous podosome formation in the absence of PDBu may be associated with activation of endogenous PKC under the culture conditions we use. Here, A7r5 cells were plated below confluence levels, where the cells have an opportunity to migrate, and it has been shown that migrating cells will exhibit activated PKCα (Ng et al., 1999). Upon PDBu treatment, 45% of cells expressing GFP-AFAP-110 showed stress filaments with no evidence for podosome formation [A], 26% of the cells show a mixture of stress fibers and podosomes [A'] and 29% display

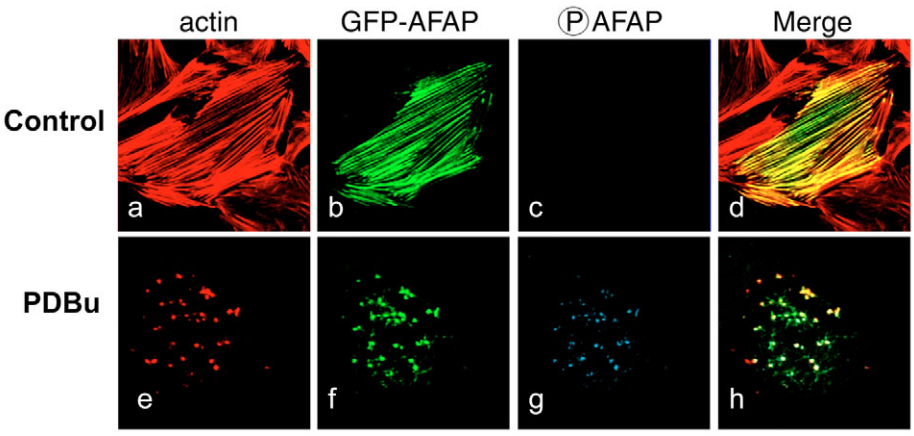

Fig. 6. Phosphorylated AFAP-110 localizes to podosomes. A7r5 cells were transfected with GFP-AFAP-110 constructs and either left untreated (panels a-d) or treated with 1 μM PDBu for 1 hour (panels e-h). Immunofluorescence staining was performed as described in Materials and Methods, with actin pseudocolored in red, GFP-AFAP-110 in green and the phosphorylation-specific AFAP-110 antibody anti-pSer277 (P-AFAP) in blue. Merged pictures show colocalization of phosphorylated AFAP-110 with AFAP-110 and actin in podosomes.

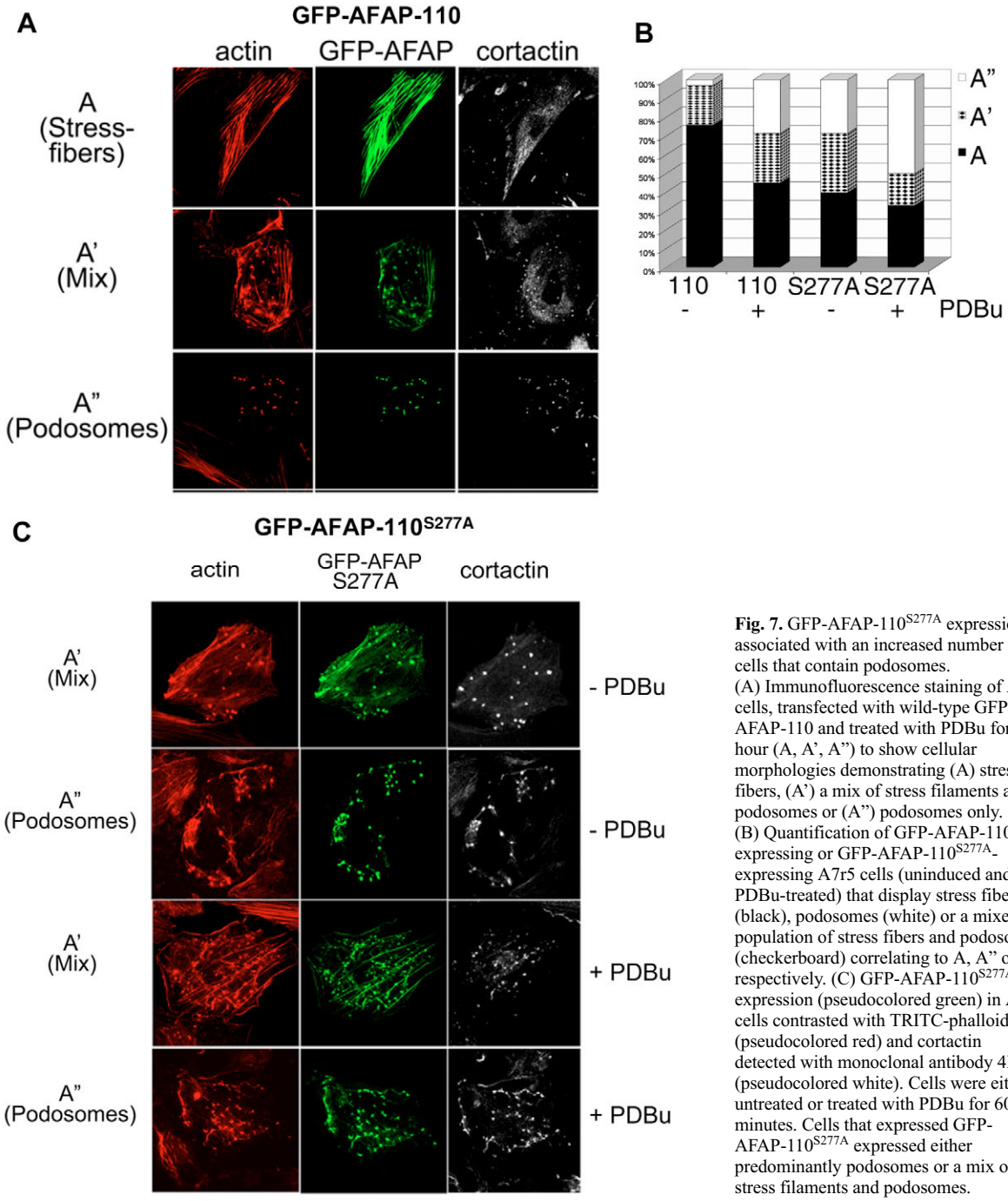

Fig. 7. GFP-AFAP-110^{S277A} expression is associated with an increased number of cells that contain podosomes. (A) Immunofluorescence staining of A7r5 cells, transfected with wild-type GFP-AFAP-110 and treated with PDBu for 1 hour (A, A', A") to show cellular morphologies demonstrating (A) stress fibers, (A') a mix of stress filaments and podosomes or (A") podosomes only. (B) Quantification of GFP-AFAP-110-expressing or GFP-AFAP-110^{S277A}-expressing A7r5 cells (uninduced and PDBu-treated) that display stress fibers (black), podosomes (white) or a mixed population of stress fibers and podosomes (checkerboard) correlating to A, A" or A', respectively. (C) GFP-AFAP-110^{S277A} expression (pseudocolored green) in A7r5 cells contrasted with TRITC-phalloidin (pseudocolored red) and cortactin detected with monoclonal antibody 4F11 (pseudocolored white). Cells were either untreated or treated with PDBu for 60 minutes. Cells that expressed GFP-AFAP-110^{S277A} expressed either predominantly podosomes or a mix of stress filaments and podosomes.

podosomes only [A"] (Fig. 7B). Podosomes were defined by their size, shape, location and colocalization with cortactin, actin and AFAP-110. Interestingly, A7r5 cells expressing AFAP-110^{S277A} showed more cells that formed podosomes (Fig. 7C). Cells expressing the phosphorylation-defective AFAP-110^{S277A} mutant showed a greater propensity to express podosomes or a mix of podosomes and stress filaments in the absence of PDBu treatment (35% stress filaments, 33% mix, 32% podosomes only), whereas PDBu treatment resulted in an increase in the number of cells that express podosomes only (30% stress filaments, 15% mix, 55% podosomes only) (Fig. 7B). These data indicate that GFP-AFAP-110^{S277A} expression correlated with an increased capacity for cells to display podosomes.

Expression of AFAP-110^{S277A} is associated with long-lived podosomes

An increase in podosome numbers might be due to increased production of podosomes or to a net decrease in the deconstruction of podosomes (extended half-life). To investigate the effect of AFAP-110^{S277A} upon podosome lifespan, we performed time-lapse video microscopy using A7r5 cells transiently expressing GFP-AFAP 110 wild type (supplementary material Movie 1A) or GFP-

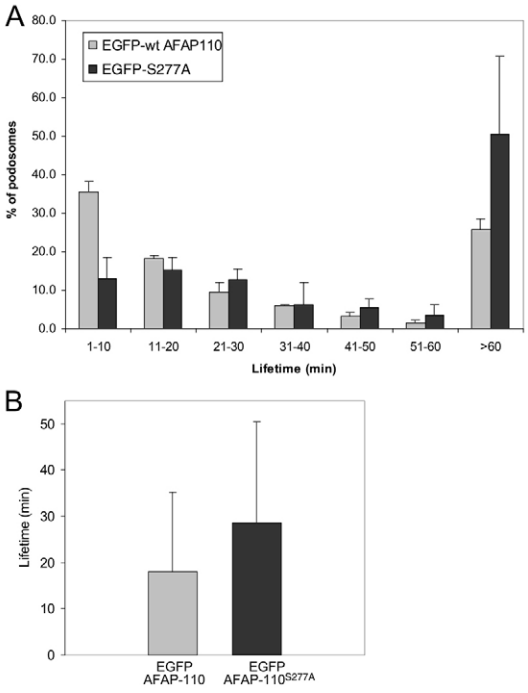

Fig. 8. GFP-AFAP-110^{S277A} expression increases the average lifespan of a podosome. (A) Percentages of podosomes were plotted against their lifespan in incremental 10-minute time brackets. The mean percentages of podosomes observed in cells from three different experiments is given ± s.d. (B) The average lifespan of podosomes was 17.9±17 minutes in cells that express GFP-AFAP 110 wild type (gray bar) and 28.6±21 minutes in cells that overexpress GFP-AFAP-110^{S277A} (black bar); the difference is statistically significant (P<0.0001).

AFAP-110^{S277A} mutant (supplementary material Movie 1B) and monitored podosome dynamics in the absence of PDBu stimulation. As shown in Fig. 8A, podosome lifespans were widely distributed from less than 10 minutes to over 60 minutes. At least half of the podosomes associated with wild-type GFP-AFAP-110 had lifespans of <30 minutes and ~25% of podosomes had a lifespan of >60 minutes (Fig. 8A, gray bars) By contrast, the distribution of the lifespans of podosomes associated with the GFP-AFAP-110^{S277A} mutation were shifted to longer time frames, with more than 50% of podosomes in a given cell having lifetimes of greater than >60 minutes (Fig. 8A, black bars). To determine the average lifetime of podosomes, a total of 361 podosomes from AFAP-110-expressing cells and a total of 477 podosomes from AFAP-110^{S277A}-expressing cells were analyzed in three independent experiments. Among those, only podosomes that appeared and disappeared during the 2-hour video microscopy were used to obtain average lifespan. As shown in Fig. 8B, expression of AFAP-110^{S277A} was associated with prolonged average lifespan of podosomes, which averaged 28.6 minutes compared with 17.9 minutes of that in wild-type-expressing cells (P<0.0001, two-tailed Student's t-test, non equal variation). These data indicate that the mutation of the Ser277 phosphorylation site to Ala is associated with an increase in the number of long-lived podosomes and a decrease of short-lived podosomes. Therefore, we conclude that the enhanced number of podosomes observed in cells expressing AFAP-110^{S277A} results from an increased lifespan of these podosomes.

Discussion

Podosomes are ventral-membrane-associated structures that promote the release of proteases, which can degrade the extracellular matrix and promote the ability of a cell to cross a tissue barrier (Linder, 2007). In general, podosomes are associated with dendritic cells, macrophages, osteoclasts, endothelial cells and smooth muscle cells, whereas oncogene-transformed fibroblast cells will form structures that resemble podosomes. A closely related cellular structure, the invadopodium, will also promote the release of proteases and the ability of a cell to cross a tissue barrier; however, invadopodia are more commonly associated with carcinoma cells. It is possible that podosomes and invadopodia are distinct structures or related structures that form on the basis of specific interactions with the substratum. Regardless, it is clear that these two structures will form in response to activation of PKCα, and that the PKCα substrate AFAP-110 is a component of these structures (Gatesman et al., 2004; Burgstaller and Gimona, 2004; Dorfleutner et al., 2007). Here, we show localization of endogenous AFAP-110 to podosomes in the PDBu-treated vascular smooth muscle cell line A7r5, which is widely used as a model system for podosome studies (Fultz et al., 2000; Brandt et al., 2002; Hai et al., 2002; Burgstaller and Gimona, 2005; Eves et al., 2006; Zhou et al., 2006). AFAP-110 consistently colocalized to podosomes and appears to be a reliable molecular marker for podosomes, where cortactin and Tks5 together with F-actin are the standard molecular biomarkers for defining podosomes on the ventral membrane of cells (Gimona et al., 2008). Our previous work has demonstrated that PKCα-directed Src activation occurs in an AFAP-110 dependent fashion, and it was this signaling cascade that was responsible for PKCα-directed podosome formation (Gatesman et al., 2004; Walker et al., 2007). Here, activated PKCα is able to bind to the PH1 domain of AFAP-110 and direct Ser/Thr phosphorylation of AFAP-110 (Qian et al., 2002), resulting in a conformational change in AFAP-110 structure that both promoted its ability to crosslink actin filaments (Qian et al., 2004) and enabled it to colocalize with and subsequently activate Src (Walker et al., 2007). Thus, we predicted that phosphorylation of AFAP-110 by PKCα may play an important role in modulating its structure and function and this may be associated with podosome formation. As AFAP-110 is phosphorylated on at least four tryptic fragments in PE-treated cells (Qian et al., 2002), we predicted that one or more of these phosphorylation sites might be relevant to its function.

In this study, we sought to identify a specific site for phosphorylation and assess whether phosphorylation of AFAP-110 may correlate with podosome formation. Preliminary in vitro studies indicated that a major site for phosphorylation of AFAP-110 by PKCα localized to amino acids 270-300 (Lidia Cherezova and D.C.F., unpublished data). This sequence contained four Ser and two Thr residues. We noticed that three of the four Ser residues (Ser277, Ser278 and Ser282) were positioned two amino acids away from positively charged Arg or Lys residues, which was defined as optimal for phosphorylation by Arg-Lys directed Ser/Thr kinases, such as PKCα (Pearson and Kemp, 1991). We noticed that, upon PE stimulation, AFAP-110 gelshifts when carrying out a 8% SDS-PAGE. Previously, we had found that AFAP-110 gelshifts in response to Src activation (which can direct PKCα activation) and that the gelshifted population was associated with a Triton-X-100-insoluble fraction of cell lysates, indicating a tight association with the cytoskeleton (Baisden et al., 2001b). Gelshifting is usually associated with Ser/Thr phosphorylation and occurs when proteins are phosphorylated on a site that is at or near the surface of a protein

whose negatively charged phosphate residue prevents negatively charged SDS from efficiently coating the protein, resulting in a retarded migration to the cathode (less-negatively charged SDS coating the protein) and an apparent increase in the molecular mass. Thus, we used gelshifting as a guide to identify a potential phosphorylation site. Mutation of Ser277 to Ala prevented AFAP-110^{S277A} from gelshifting in response to PE treatment. Thus, we predicted Ser277 to be a phosphorylation site and obtained an antibody that specifically recognizes AFAP-110 phosphorylated at Ser277. This phospho-specific antibody (anti-pSer277) recognized only one major protein band among proteins obtained from cell lysates and separated by 8% SDS-PAGE and immunoreactivity was specifically associated with phosphorylation of Ser277. Immunoprecipitation of AFAP-110 and western blot analysis with anti-pSer277 confirmed its specificity for AFAP-110 and, as predicted by sequence analysis, it was crossreactive with the avian, human and rodent homologs of AFAP-110. Thus, we conclude that Ser277 is a site for phosphorylation in AFAP-110 in response to PE stimulation.

Interestingly, phosphorylation of AFAP-110 in response to PE treatment was dependent upon the presence of the PH1 domain. Previous work by our lab demonstrated that the PH1 domain is a site for binding by the PKC α, β, γ and λ isoforms (Qian et al., 2002). Furthermore, AFAP-110 can relay signals from PKCα that direct Src activation; however, deletion of the C-terminal half of the PH1 domain prevented PKCα from directing Src activation. Thus, we predict that PKCα binds to the PH1 domain of AFAP-110 as a prelude to phosphorylating AFAP-110. It is interesting that the N-terminal PH domain in the protein pleckstrin will also bind to PKCα, and that phosphorylation of Ser113 and Ser117, which like Ser277 occurs, are within 25 amino acids of, and proximal to, the termination of their PH domains (Craig and Harley, 1996; Yao et al., 1997). Thus, it might be possible that the binding of PKCα to a PH domain orients it such it can phosphorylate a specific Ser/Thr residue adjacent to the PH domain. The ability of PEs to stimulate phosphorylation of AFAP-110 at Ser277 was not dependent upon the ability of AFAP-110 to bind to Src. Pro71 is a key component of the left-handed, type II poly-proline helix that engages the Src SH3 domain (Guappone and Flynn, 1997). Binding to Src through this motif will serve to activate Src (Baisden et al., 2001a). As Src will in turn activate other Ser/Thr kinases (including PKCα), we infer from these data that PE-directed phosphorylation of Ser277 occurs upstream and independent of Src activation. This is confirmed with the addition of PP2, which failed to block AFAP-110 phosphorylation on Ser277 in response to PE treatment. It seems surprising that actin filament binding was not required for phosphorylation of Ser277, indicating that strict positioning upon the cytoskeleton was not a prerequisite for phosphorylation of AFAP-110 in response to PE treatment.

PEs activate a variety of signaling proteins in addition to classical and novel PKC family members (Kazanietz, 2000). Thus, it is possible that a kinase other than PKC was directing phosphorylation of AFAP-110 on Ser277. To begin to address this, we utilized broad-spectrum inhibitors that have some specificity for PKC family members, as well as other Ser/Thr kinases (Davies et al., 2000). Staurosporine and Safingol have some specificity for PKCα as well as other Ser/Thr kinases and were able to block PMA-directed phosphorylation of Ser277. Rottlerin can block the activity of some Ser/Thr kinases, but does is not specific for PKCα and was unable to block phosphorylation of Ser277 in response to PE treatment. In addition, expression of myrPKCα directed phosphorylation of Ser277, and GF109203X – which has reasonable specificity for PKCα – blocked PE-directed phosphorylation of Ser277. Collectively, these data are consistent with PKCα being responsible for mediating phosphorylation of Ser277 in response to PE treatment. Thus, we hypothesize that PE-directed activation of PKCα directs it to bind to the PH1 domain of AFAP-110 and phosphorylate it directly on Ser277.

We assessed the functional implications of phosphorylation of Ser277. Anti-pSer277 localized phospho-AFAP-110 to podosomes, although some immunoreactivity was apparent on stress filaments in cells that showed a mix of podosomes and stress filaments, which had formed in response to PE treatment (data not shown). Anti-pSer277 was not able to efficiently detect endogenous phospho-AFAP-110. This might be due to lower protein expression levels (relative to transfected cells) and that only a fraction of the endogenous proteins is phosphorylated, placing immunoreactivity of endogenous phospho-AFAP-110 at or below detection limits. As ectopically expressed GFP-AFAP-110 aligned with stress filaments and podosomes in a fashion that is consistent with that of endogenous protein, we assessed the role of GFP-AFAP-110^{S277A} in podosome formation relative to GFP-AFAP-110. It is noteworthy that GFP-AFAP-110^{S277A} localizes to podosomes as well as GFP-AFAP-110. Thus, phosphorylation of AFAP-110 on Ser277 is not required for podosome localization to occur. However, we noticed that a substantial number of unstimulated A7r5 cells that expressed GFP-AFAP-110^{S277A} contained podosomes, whereas only a small fraction of the unstimulated A7r5 cells that expressed GFP-AFAP-110 had podosomes. Furthermore, upon stimulation with PDBu, a greater proportion of A7r5 cells that expressed GFP-AFAP-110^{S277A} had podosomes relative to those cells that expressed GFP-AFAP-110. We believe that the presence of podosomes in unstimulated cells is linked to our cell culture conditions, because we plated and observed the A7r5 cells at subconfluent concentrations. Under these conditions, the cells can be motile and it has been shown that PKCα will become activated during cell migration (Ng et al., 1999), indicating a possible mechanism by which podosomes may form in unstimulated cells. The number of podosomes associated with GFP-AFAP-110^{S277A} expression was substantially higher, indicating either an increased rate of podosome formation or increased podosome stability. Thus, we used video microscopy to analyze podosome half-life. We showed that expression of AFAP-110^{S277A} leads not only to an increased number of total cells with podosomes but also to an increase in the number of long-lived podosomes. Thus, it is possible that phosphorylation of Ser277 is important for the deconstruction of podosomes, and that loss of this phosphorylation site results in podosomes with a longer half-life. Also possible is that long-lived podosomes are different compared with short-lived podosomes. We did notice a trend that indicated long-lived podosomes were larger than short-lived podosomes, which is the subject of an ongoing study in our lab (Y.C. and D.C.F., unpublished). In general, podosomes have a relatively short half-life (~10 minutes or less). Interestingly, invadopodia have a half-life of 2-3 hours and can be significantly larger than short-lived podosomes (Linder, 2007). Thus, it is possible that phosphorylation and dephosphorylation of AFAP-110 has a role in fostering dynamic changes in size and longevity of podosomes.

We hypothesize that phosphorylation of AFAP-110 on Ser277 promotes deconstruction of podosomes and this may be relevant for podosome half-life. There is a subset of proteins that are thought to have a role in regulating podosome half-life, and that may be important regulators of podosome structure and function. Recently

it has been shown that inhibition of Erk1/2 phosphorylation leads to increased podosome lifetime in PDBu-stimulated A7R5 cells (Gu et al., 2007). Through detailed mathematical modeling, it has been shown that increased podosome lifetime was mainly affected by the decreased rate constant for podosome disassembly, which indicated that Erk1/2 phosphorylation is involved in facilitating podosome disassembly. In that regard, it is possible that phosphorylation of Ser277 of AFAP-110, similar to Erk1/2 phosphorylation, is another physiologically relevant event that can promote disassembly of podosomes. Another protein that could regulate podosome half-life is cofilin, because knockdown of cofilin results in decreased invadopodia half-life (Yamaguchi et al., 2005). It has been speculated that this property of cofilin makes it one of the master switches that promote transition of podosomes to invadopodia (Linder, 2007). AFAP-110 might have similar properties. Finally, it is also possible that AFAP-110, as a protein that regulates cell contractility and crosslinks stress filaments, regulates podosome half-life through these functions. Podosomes are adhesive structures and their formation might be dependent upon contractility. To this end, we have noticed the presence of myosin II in the ring of podosomes associated with AFAP-110^{S277A}, similar to that observed by others in podosomes (Burgstaller and Gimona, 2004; van Helden et al., 2008), and reminiscent of the localization pattern of vinculin (supplementary material Fig. S2). It is possible that phosphorylation of AFAP-110 affects a change in actin filament crosslinking, interactions with myosin II and contractility; however, there is not an apparent change in myosin II localization around podosomes that express AFAP-110^{S277A}, compared with that described in previous reports. It is noteworthy that, in vitro, recombinant AFAP-110 exists as a multimer (it is predicted to be a nonamer) and that in this form it is able to promote organizational changes with actin filaments that have been predicted to exist in meshworks. However, upon phosphorylation by PKCα, AFAP-110 assumes a smaller multimeric existence (possibly that of a trimer), which is able to promote tight packing of stress filaments and has been predicted to be an efficient F-actin-bundling protein (Qian et al., 2004). Thus, phosphorylation of AFAP-110 and subsequent dephosphorylation might affect changes in conformation that alter stress filament crosslinking patterns, and this ability might promote changes in actin filament architecture in response to contractility changes. Studies are underway to determine the effect of Ser277 phosphorylation on the actin filament crosslinking properties of AFAP-110. However, our hypothesis is that AFAP-110^{S277A} is unable to transition from an actin-meshworking to an actin-filament-crosslinking protein and this inability might impede contractility signals that promote deconstruction of podosomes in response to changes in cellular signals or cell adhesion. Since AFAP-110 is positioned at specialized microdomains at the interface between focal adhesion and stress fiber – the origin of podosome formation (Burgstaller and Gimona, 2004) – AFAP-110 would seem to be well positioned to have a role in podosome formation. We are actively investigating the exact molecular mechanisms through which the phosphorylation of AFAP-110 leads to podosome disassembly.

Materials and Methods

Cell culture and reagents

A7r5 rat vascular smooth muscle cells were purchased from the American Type Culture Collection (ATCC) and grown in low-glucose (1 g/l) Dulbecco's modified Eagle's medium (DMEM; Mediatech) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 2 mM L-glutamine (Gibco), and penicillin/streptomycin (Mediatech) at 37°C with 5% CO_2. A7r5 cells were transfected with Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's protocol. The COS-7 cell line was grown in high-glucose (4.5 g/l) DMEM (Mediatech), 10% FBS, 2 mM L-glutamine and penicillin/streptomycin at 37°C with 5% CO_2. For transfection of COS-7 cells Polyfect (Qiagen) was used according to the manufacturer's protocol.

The following antibodies were used for western blotting or immunofluorescence: anti-GFP (Invitrogen), anti-AFAP-110 [first described by Flynn et al. (Flynn et al., 1993)], anti-cortactin (4F11 from Upstate), tetramethylrhodamine-isothiocyanate (TRITC)-conjugated phalloidin (Sigma). The antibody specific against phosphorylated AFAP-110 was generated by 21st Century Biochemicals. Briefly, rabbits were immunized repeatedly with AFAP-110 peptide (encoding amino acids 273-283) phosphorylated at Ser277. Antibody reactivity was tested by ELISA and phosphorylation specificity was initially confirmed in a dot-blot assay. Non-phosphorylated or phosphorylated peptides were suspended in water and 1 µl of aliquots of different peptide concentrations (5-1200 ng) were spotted on a nitrocellulose membrane that was hybridized with rabbit antiserum (1:1000). Subsequently, the phosphospecific antibody was affinity-purified by affinity absorption against the phosphorylated peptide by 21st Century Biochemicals.

The PKC inhibitors staurosporine, rottlerin and Safingol as well as the Src-specific inhibitor PP2 were purchased from EMD Biosciences.

Constructs

The following constructs have been described previously : GFP-AFAP-110 and GFP-AFAP-110 ΔPH (Qian et al., 2002), GFP-AFAP-110^{P71A} (Guappone and Flynn, 1997), GFP-AFAP-110$^{1-616}$ and GFP-AFAP-110$^{1-553}$ (Qian et al., 2000). To eliminate the potential Ser phosphorylation sites at positions 277, 278, 282 and 283 the GFP-AFAP-110 construct was subjected to single or multiple rounds of site-directed mutagenesis using the Stratagene QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's recommendations., resulting in the 4A mutant (S277A-S278A-S282A-S283A), S277A-S278A and S282A-S283A double mutants, or S277A and S278 single amino acid mutants. HA-AFAP was generated by PCR amplification and cloned into pCMV-HA. All constructs were confirmed by sequence analysis. PKCα expression constructs were a kind gift from Alex Toker (Israel Deaconess Medical Center, Boston, MA) and have been described previously (Gatesman et al., 2004).

Immunoprecipitation and immunoblotting

For phorbol-12-myristate-13-acetate (PMA, EMD Biosciences) treatment of cells, PMA was used at 100 nM for 1 hour if not indicated otherwise. In overexpression experiments cells were directly lysed in equal volumes of Laemmli sample buffer and equal volumes were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For immunopreciptiation of endogenous AFAP-110, cells were lysed for 20 minutes on ice in RIPA lysis buffer (10 mM Na_2PO_4 pH 7.2, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 5 µg/ml aprotinin, 2 µg/ml leupeptin, 1 mM PMSF, 100 mM sodium fluoride, 10 mM β-glycerophosphate, 100 µM sodium orthovanadate), sonicated briefly and cleared by centrifugation at 4°C for 10 minutes at 12,000 ***g***. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce) according to the manufacturer's protocol. Equal protein amounts were used for immunoprecipitation with 2 µg AFAP-110 antibody overnight at 4°C followed by incubation with protein A sepharose (Sigma) for 1 hour at 4°C. Immune-complexes were washed twice with RIPA lysis buffer and once with phosphate-buffered saline (PBS) before SDS sample buffer was added and SDS-PAGE was performed. Resolved proteins were transferred by semi-dry electroblotting to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P from Millipore). Proteins were detected by incubation with primary antibodies as indicated, in a solution of 5% (w/v) powdered milk, 0.05% (v/v) Tween 20 in TBS, followed by incubation with a 1:3000 dilution of secondary donkey anti-mouse or donkey anti-rabbit horseradish-peroxidase-conjugated antibodies (GE Healthcare Bio-Sciences). Peroxidase-conjugated secondary antibodies were visualized by enhanced chemiluminescence. To re-probe membranes the antibodies were stripped off the membrane by incubation at 65°C in SDS-stripping buffer for 30-60 minutes.

Immunofluorescence

Cells were seeded on 10 µg/ml fibronectin-coated coverslips and grown overnight. Where indicated cells were treated with 1 µM phorbol-12,13-dibutyrate (PDBu; EMD Biosciences) for 1 hour, then fixed in 3.7% formaldehyde, permeabilized with 0.2% Triton X-100 and stained for actin with TRITC-phalloidin and polyclonal AFAP-110 antibody. Secondary antibodies (Invitrogen) were used at a 1:200 dilution. Coverslips were mounted on slides with Fluoromount-G (Southern Biotech) and pictures were taken with a Zeiss LSM 510 microscope. Images were imported into Photoshop 7.0 (Adobe) for processing.

Matrix-degradation assay

ECM degradation assays were performed as described by Burgstaller and Gimona (Burgstaller and Gimona, 2005) with modification. A glass coverslip was coated with poly-L-Lysine and crosslinked with 2.5% glutaraldehyde. Oregon Green 488 gelatin (Sigma) was mixed with 50 ug/ml fibronectin in 2 M urea buffer in PBS and layed over the poly-L-lysine-coated coverslip. After quenching with borohydride (5 mg/ml), the coverslip was washed, sterilized with ethanol, and then equilibrated with complete

medium. Cells were allowed to degrade the gelatin and fibronectin mixture for 16 hours. Images were obtained from an Axiovert 200M inverted microscope.

Time-lapse analysis of podosomes in A7r5 overexpressing wt AFAP-110 or AFAP-110S277A

A7R5 were transiently transfected with EGFP-AFAP-110 or EGFP-AFAP-110^{S277A} mutant using Lipofectamine and PLUS reagent (Invitrogen). After a 24-hour incubation, the transfected cells were plated on Delta T dishes (Bioptechs Inc) pre-coated with 50 μg/ml fibronectin and allowed to adhere for 24 hours. Medium was replaced with DMEM containing 25 mM HEPES without Phenol Red and supplemented with 10% FBS before each videomicroscopy analysis. Cells with EGFP AFAP-110-positive podosomes were videotaped at 37°C for up to 3 hours, using a Nikon Swept-field inverted microscope equipped with a CoolSNAP HQ_2 16-bit camera (Photometrics) or a Zeiss Axiovert 200M inverted microscope. The acquired images were analyzed with ImageJ (NIH image) with MTrackJ and ParticleTracker Plugins. Duration, intensity and net speed of each podosome were recorded. When a podosome underwent fission, the daughter podosome with brighter intensity was regarded as the continuing podosome and the other daughter podosome as a new podosome. To obtain the average lifetime of podosomes, only the lifespan of podosomes that had appeared and had disappeared during videomicroscopy were used. The two-tailed Student's *t*-test with non-equal variance was used to determine the statistical significance of duration difference between podosomes containing wild type and 277A mutant of AFAP-110.

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